Studies on Properties and Roles of Thymocytes and T-lymphocytes in Tumor Cell Destruction and T-cell Deficiency Mouse(癌細胞の破壊及びT細胞不全マウスにおける胸腺とTリンパ球の役割及び特性に関する研究)

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URL	http://hdl.handle.net/10097/46019

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by

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CONTENTS

I. Introduction

The immune system consists of lymphocytes, macrophages, dendritic cells and epidermal Langerhans' cells. Lymphocytes differ from one another both phenotypes and functional properties. Two broad lineages of lymphocytes are recognized: the B lymphocytes, which are precursors of antibody-secreting cells, and the T, or thymus-dependent, lymphocytes. T lymphocytes have both regulatory and effector functions and are particularly important in "cell-mediated immunity", including reactions to microorganisms that are obligate intracellular parasites, in graft rejection, and in immune responses to tumor. As already noted, T lymphocytes also play an essential role in antibody responses by helping B lymphocytes to differentiate into antibody-producing cells. However, the details of the activation mechanism of T lymphocytes involving cell surface molecules are still unclear.

T lymphocytes derived from stem cells located within hematopoietic tissues. Such T cell precursors migrate to the thymus where they undergo an ordered differentiation process (Fig. 1-1). The most immature thymocytes are initially found in the peripheral part of the thymic cortex. These cells fail to express both CD4 and CD8 markers and are referred to as "double negative" cells. Most of double negative thymocytes differentiate into the cells expressing both CD4 and CD8 cell surface antigens, "double positive" cells. A part of double positive thymocytes undergo both self-tolerance induction and positive selection (determination of self-MHC-restriction) and become $CD4^+$, $CD8^-$ cells or into $CD4^-$, $CD8^+$ ("single positive") cells. These single positive cells have been considered to be maturated immunocompetent T cells and are capable of entering the peripheral lymphoid organs. These differentiated thymocytes migrate to the T-dependent area of peripheral lymphoid organs such as lymph nodes, spleen and Peyer's patches of the intestine through the high endothelial venules (HEV). The details for releasing or migration mechanisms of these thymocytes are unclear.

On such a background, development of T cell-mediated immunity was further analyzed in the present study from the two standpoints; roles of cell surface accessory molecules in T cell activation mostly working in the field of tumor immunology and establishment of a new animal model for T

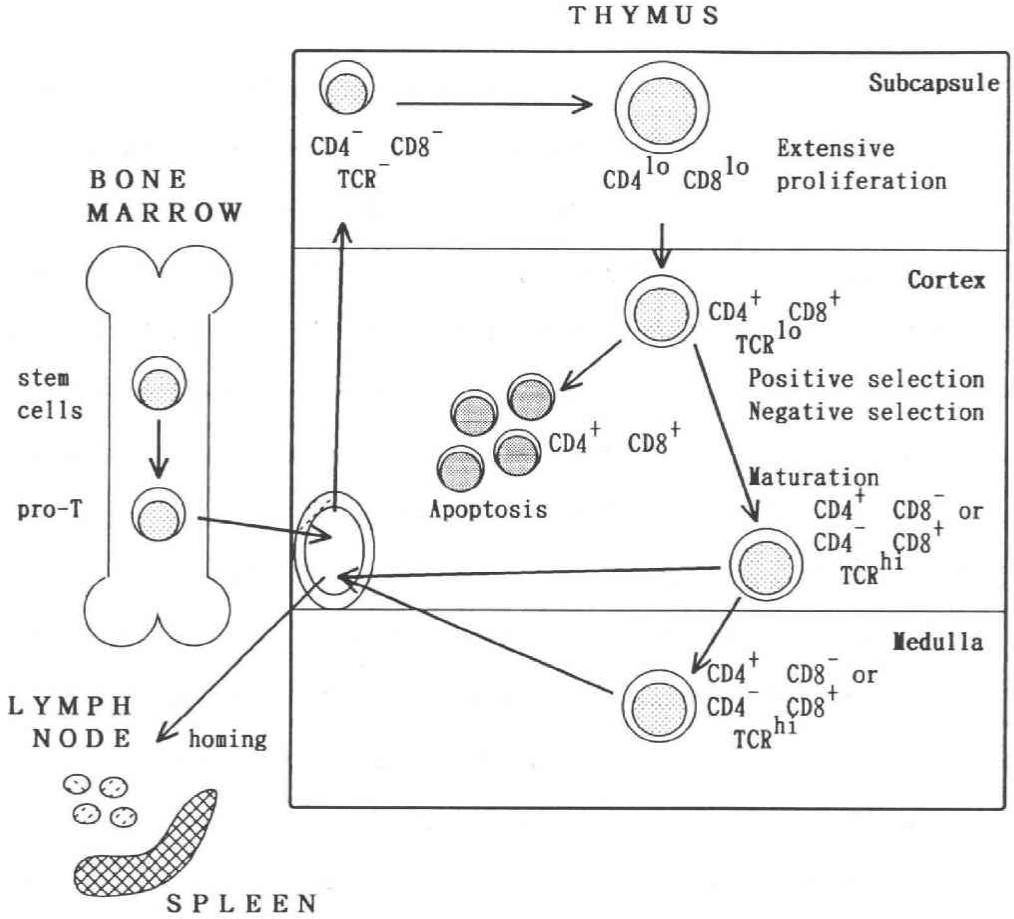

Fig. 1-1. Differentiation of thymocytes

cell-deficiency.

As for tumor immunology, theoretically, the outgrowth of primary cancer seems to be prevented by immune response, as indicated by the Burnet's immunological surveillance concept (1). However, immune system cannot aggressively respond to the great mass of tumor except for a part of benign one. Nevertheless immunotherapies have been performed with the following methods: 1) Stimulation or activation of immune systems against tumor cells by use of vaccine or biological response modifiers (BRM), 2) adoptive transfer of clonal killer cells including killer T, NK, or LAK

cells, 3) chemoimmunotherapy using toxin- or drug-conjugated monoclonal antibody, and 4) bone marrow transplantation in leukemia patients after elimination of tumor cells or T cells in bone marrow cells by treating with monoclonal antibody.

Recently, the advent of gene technology enabled us to obtain a various kind of recombinant cytokines. Although interleukin 2 (IL-2) was first discovered as a T cell growth factor (TCGF) (2, 3), more recent studies showed that it has multi-functions, e.g., stimulation for proliferation of T, B and NK cells, differentiation of T and B cells, augmentation of non-MHC-restricted cytotoxicity of NK and T cells, and activation of macrophages (4-6). Murine lymphocytes incubated with recombinant IL-2 (r-IL-2) generate lymphokine-activated killer (LAK) cells that are able to lyse a wide variety of fresh, NK-resistant tumor cells (7-9). Thus, LAK cells are applicable for clinical cancer therapy, because LAK cells can be induced easily and can lyse a variety of malignant tumor cells but not normal cells. Before the therapeutic application of LAK cells, I characterized the properties of LAK cells in their precursor and effector phases (Chapter II).

Of interest are the molecules which may be involved in the cell-killing mechanisms of broad-reactive killer (BRK) cells including LAK cells. I recently established hybridoma cells producing a mAb (termed KBA) which is able to block the cytotoxicity of BRK cells, including LAK cells, NK cells and activated macrophages, but not cytotoxic T lymphocytes (CTL) (10). I designated the antigen to which this mAb is directed the lymphokine-activated cell-associated (LAA) antigen. The LAA antigen seems to be one of the accessory molecules, because KBA mAb can block the binding of LAK cells to target tumor cells and the MW of LAA antigen is similar to that of LFA-1. In this paper, I investigated the correlation between the LAA antigen expression and the in vitro and in vivo activation of lymphocytes or the cell cycle (Chapter III).

The investigation of the role of cell surface molecules involved in the recognition and the activation of T lymphocytes is an important issue for the evaluation of the mechanisms of immune regulatory system. Triggering of T lymphocytes via T cell receptor (TCR) is one of the most important step in the activation of T cells. However, the development of monoclonal antibodies

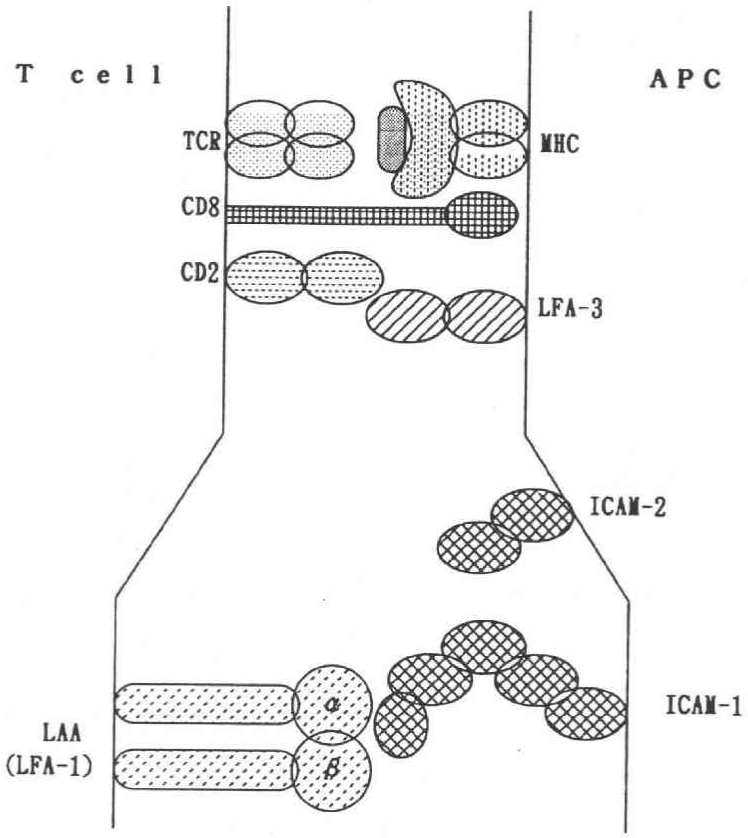

Fig. 1-2. Interaction of adhesion molecules[93]

(mAbs) to the cell surface molecules on immunoregulatory cells has suggested that several additional surface molecules on T cells or antigen-presenting cells (APC) are required for the stable activation of T cells (Fig. 1-2). Major histocompatibility complex (MHC) class I or class II, CD4 (L3T4), CD8 (Lyt2), CD2 (LFA-2), CD58 (LFA-3), CD54 (ICAM-1) and CD11a.CD18 (LFA-1) are all well known functional accessory molecules (11-19). However, the precise role of these accessory molecules is still unclear. In this paper, I investigated the role of LAA antigen, one of the functional accessory molecules, on T cell activation by mitogens, allo-antigen, auto-antigen or IL-2 (Chapter

IV).

For the study of T lymphocyte properties, T-cell deficiency mice are useful. Nude mice which are hereditary athymic are frequently used as a model of T cell-deficient animal (20). However, establishment of other type of T cell-deficient model,if any, will present more informations on the generation and properties of T lymphocytes. Fortunately, Shionogi Aburahi Laboratories has developed and maintained an inbred mouse strain named cataract Shionogi (CTS), which shows cataract eyes with microphthalmia and is euthymic but deficient in T cell functions. I evaluated the usefulness of CTS mice as the model: first, the immunological characteristics of this mouse strain was studied especially paying attention to its T cell functions, and second, the causes of T-cell deficiency was analyzed. In this paper, I describe the several in vitro immune responses and cell surface phenotypes of the lymphocyte populations in the spleen (Chapter V) and the in vivo immune responses against thymus-dependent and -independent antigens (Chapter VI).

II. Characteristics of Mouse Thymocyte-derived, Interleukin-2-activated Killer Cells and Their Precursors

INTRODUCTION

Lymphokine-activated killer (LAK) cells, which are distinct from cytotoxic T lymphocytes and natural killer (NK) cells in their cell surface phenotype and target specificity, are inducible from lymphoid cells of experimental animals and humans by culture with interleukin 2 (IL-2) (7, 9, 21). These LAK cells exhibit strong cytotoxicity against a variety of tumor cells not only in vitro but also in vivo (22-25). Since "LAK" is a general term for the lymphokine-activated killer cell activity, LAK cells appeared to be inducible from multiple lymphoid cell populations and involve cell populations that have different phenotypes. Indeed, as I have demonstrated earlier (21, 26, 27), the cell-surface phenotypes of LAK cell populations induced from normal mouse spleen cells, nude mouse spleen cells and tumor-infiltrated lymphocytes were common in the expression of Thy 1 antigen and lymphokine-activated cell-associated antigen (LAA; corresponding to LFA-1 in molecular character and antigen distribution (10)) but different in the expression of CD3, Lyt 2 (CD8) antigens and asialoGM_1.

Thymocytes have been regarded as immunoincompetent cells, but recent studies have shown that hydrocortisone (HC)-resistant, peanut agglutinin receptor-negative (PNA^-) large thymocytes are able to respond to various antigens, as opposed to small thymocytes, and that IL-2 stimulation leads these thymocytes to LAK cells (28-32). Although Budd et al. (32) have demonstrated recently that stimulation of Lyt 2 $(CD8)^-$, L3T4 $(CD4)^-$ T cells from mouse thymus with culture supernatant containing IL-2 activity resulted in the induction of killer cells having a broad target spectrum, the properties of LAK from thymocytes and their precursors remained to be studied in detail. I induced LAK cells from mouse thymocytes by using recombinant human IL-2 (rh-IL-2) and studied the phenotypes of LAK cells and their precursors. This paper reports that rh-IL-2 allows the efficient

induction of Thy 1^+, Lyt 2^+, asialoGM_1^-, high-density LAA-bearing LAK cells from HC-resistant mouse thymocytes (HCRT) and that their precursor cells are Lyt 1 $(CD5)^-$ or very-low-density (dull) Lyt 1^+, L3T4$^-$, Lyt 2^- large thymocytes.

RESULTS

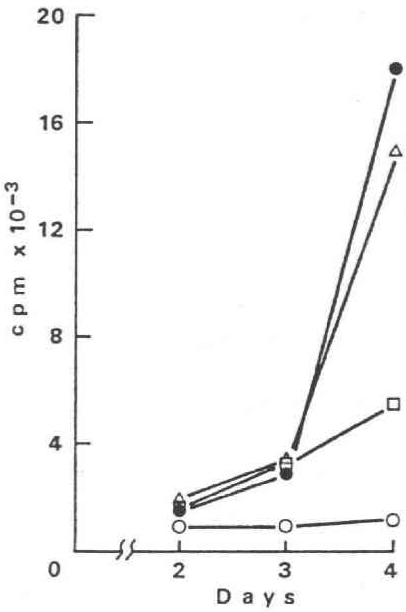

Fig. 2-1. Effect of hydrocortisone (HC) treatment on IL 2 responsiveness of C57BL/6 mouse thymocytes. Thymocytes were obtained before or 24 h after an intraperitoneal injection of a suspension of hydrocortisone acetate in physiological saline. Untreated or HC-treated mouse thymocytes (5×10^5) were cultured with 2,000 JU/ml of rh-IL-2 for 4 days and pulsed with ^{3}H-thymidine (0.5 μCi/well) for 4 h. Dose (mg/mouse) of HC: ○, 0; ●, 1.25; △, 2.5; □, 10.

I first examined the response of HC-pretreated and untreated C57BL/6 mouse thymocytes to rh-IL-2. Since, in the preliminary experiments, I found that the IL-2 dose (500 to 1,000 JU/ml) required for the proliferation of, and induction of LAK cells from, spleen cells was not enough to

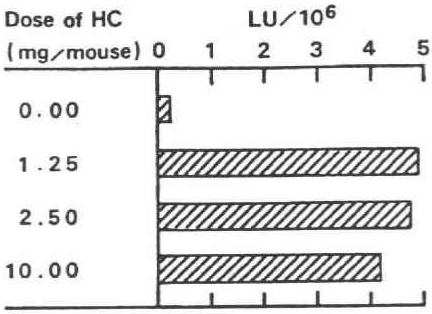

Fig. 2–2. Effect of HC treatment on LAK generation from thymocytes. Untreated or HC–treated C57BL/6 mouse thymocytes (5 x 10^6) were cultured with rh–IL–2 (2,000 JU/ml) for 4 days and then their cytotoxic activity against BMC2 tumor cells was determined by the ^{3}H–uridine method. Lytic unit (LU)/10^6 cells was calculated as described in "Materials and Methods".

stimulate thymocytes, 2,000 JU/ml of IL–2 was employed in the present study. The proliferative response of thymocytes to rh–IL–2 is shown in Fig. 2–1. Normal thymocytes cultured for 4 days in the presence of rh–IL–2 (2,000 JU/ml) showed only a small proliferative response. However, when mice were pretreated with HC at a dose of 1.25 mg/mouse, the HCRT showed a large proliferative response to rh–IL–2. A similar response was observed with the thymocytes from mice treated with 2.5 mg of HC, but the response of the thymocytes from mice treated with 10 mg of HC was lower than that. In accordance with these results, killer cells strongly cytotoxic to syngeneic BMC2 fibrosarcoma cells were generated from HCRT, but not from untreated mouse thymocytes (Fig. 2–2).

I next examined the target specificity and phenotypes of LAK cells generated from HCRT. These LAK cells were cytotoxic to a variety of mouse sarcoma and lymphoma cells of different H–2 haplotypes (Fig. 2–3). In order to assess cell surface phenotype, the LAK cells were treated with antibodies to cell surface alloantigens in the presence of complement and tested for cytotoxicity to BMC2 cells. The cytotoxic activity of the LAK cells was diminished by treatment with either anti–Thy 1.2 antibody or anti Lyt 2.2 antibody, but not with anti–asialoGM_1 antibody (Fig. 2–4). These results indicate that LAK cells generated from HCRT are Thy 1^+, Lyt 2^+, asialoGM_1^- T cells.

For further characterization, the LAK cells induced from HCRT were fractionated by Percoll

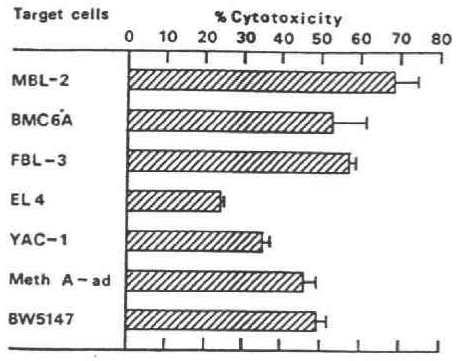

Fig. 2-3. Target specificity of LAK cells from hydrocortisone-resistant thymocytes (HCRT). HCRT (5 x 10^6) were cultured with 2,000 JU/ml of rh-IL-2 for 5 days and their cytotoxicity against various kinds of tumor cells was determined by the ^{3}H-uridine method. Effector:target=50:1.

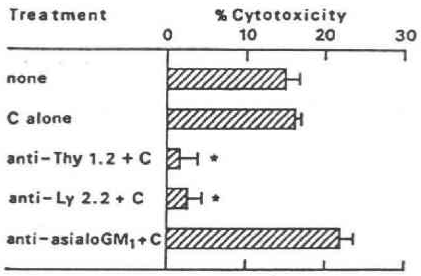

Fig. 2-4. Negative selection of LAK cells generated from hydrocortisone-resistant thymocytes (HCRT). LAK cells from HCRT were treated with alloantibody and complement and tested for cytotoxicity against BMC2 tumor cells by the 3H-uridine method (effector:target=25:1). Statistical significances were determined by Student's *t*-test; * $p<0.05$.

discontinuous density gradient centrifugation and cells in each fraction were assessed for phenotype, cytotoxic activity and capacity for target cell binding (Table 2-1). The LAK cells were fractionated into four subpopulations with different densities (Fr-1 to Fr-4). The cytotoxicity and target cell-binding assays using BMC2 and MBL-2 cells revealed that the cell populations of lower density and larger size in Fr-1 (54% of total) and Fr-2 (13% of total) showed significant target cell-binding and cytotoxicity, whereas the other subpopulations with higher density and smaller size in Fr-3 (10% of total) and Fr-4 (23% of total) showed a little target-binding capacity and no cytotoxicity. These LAK

Table 2–1

Cytotoxicity and binding capacity to target cells of LAK cells fractionated by Percoll density gradient

LAK cells[a]	Cytotoxicity[b] (LU/10^6 cells)	% Binding[c] to	
		BMC2	MBL–2
Unfractionated	1.87	23.7	19.8
Fraction 1	2.29	25.7	29.5
Fraction 2	1.74	29.0	25.4
Fraction 3	<0.04	4.2[d]	4.8[d]
Fraction 4	<0.04		

a) LAK cells were induced from C57BL/6 mouse thymocytes by culture with rh–IL–2 and fractionated as described in "Materials and Methods". Fraction 1 to 4 cells represent those cells that accumulated on the layers of 40, 55, 60 and 80% Percoll solutions, respectively.

b) Cytotoxicity against BMC2 tumor cells was measured as described in " Materials and Methods".

c) More than 200 fluorescently labeled cells were observed for binding to target cells.

d) Fractions 3 and 4 cells were combined and used for assay.

cell subpopulation were then examined for cell surface phenotypes by flow cytometry. Activation of HCRT with rh–IL–2 resulted in a small increase of Thy 1 and Lyt 2 and a small decrease of Lyt 1 (data not shown). In contrast, it evoked a marked increase of LAA (Fig. 2–5). As to Lyt 2 expression, larger size thymocyte subpopulations of stronger LAK activity (Fr–1 and Fr–2) were found to express a greater amount of Lyt 2 antigen as compared to the smaller size thymocyte subpopulations (Fr–3 and Fr–4) (Fig. 2–6).

I next investigated the LAK cell precursors in thymocytes. The frequency of LAK cell

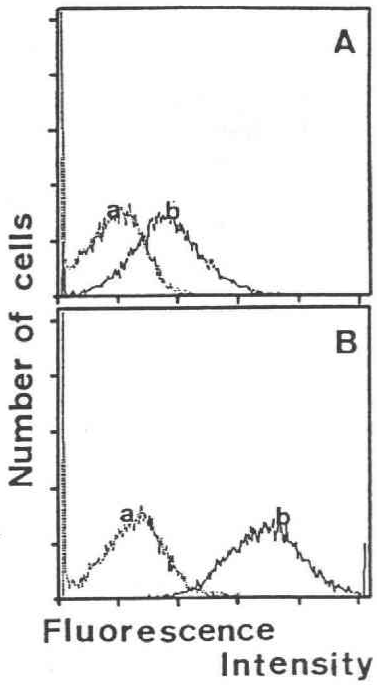

Fig. 2–5. Augmented expression of LAA antigen in IL–2–treated hydrocortisone–resistant thymocytes (HCRT). HCRT or rh–IL–2 (2,000 JU/ml)–stimulated HCRT were treated either phosphate–buffered saline containing 1% bovine serum albumin (BSA–PBS) or FITC–conjugated anti–LAA antibody (KBA) and assayed for fluorescence intensity by flow cytometry. A, HCRT; B, IL–2–stimulated HCRT. a, BSA–PBS; b, FITC–KBA.

precursors in normal thymocytes and in HCRT were assayed by a limiting dilution technique. The frequency of LAK precursors was 7.5 times greater in HCRT than in total thymocytes (Table 2–2), suggesting that the in vivo HC treatment led to concentration of the precursor cells in thymocytes and thus enabled the efficient induction of LAK cells by culture with IL–2.

I finally examined the phenotypes of LAK precursor cells. Prior to the activation with IL–2, HCRT were treated with anti–alloantigen antibody and complement and then assessed for capacity for the induction of LAK cells. The treatment of HCRT with anti–Lyt 2 antibody plus complement or anti–L3T4 antibody plus complement resulted in a complete elimination of thymocytes possessing

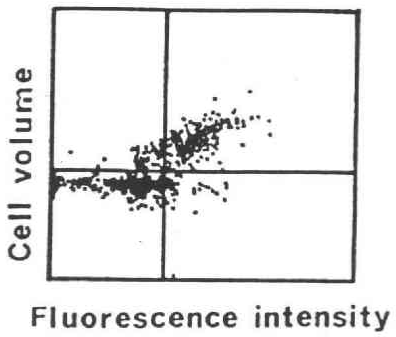

Fig. 2-6. Flow cytometric analysis of Lyt 2 antigen on LAK cells generated from hydrocortisone-resistant thymocytes. The figure represents the fluorescence intensities and cell volumes of 1,000 individual cells.

Table 2-2

Frequency of LAK cell precursors in untreated and hydrocortisone-treated C57BL/6 mouse thymocytes

Treatment	No. cells/ thymus ($\times 10^6$)	Frequency of precursors	No. precursors/ thymus
Untreated	110.0	1/15,000	7,300
Hydrocortisone (2.5 mg/mouse)	8.8	1/2,000	4,400

these alloantigens, as determined by flow cytometry. In contrast, treatment with anti-Lyt 1 antibody plus complement could eliminated high intensity Lyt 1-bearing (bright Lyt 1^+) T cells, but not low intensity Lyt 1-bearing (dull Lyt 1^+) T cells from the HCRT (data not shown). However, the pretreatment of HCRT with anti-Lyt 1 antibody or anti-Lyt 2 antibody or anti-L3T4 antibody and complement did not affect the IL-2-dependent LAK cell induction (Fig. 2-7). Moreover, culture of

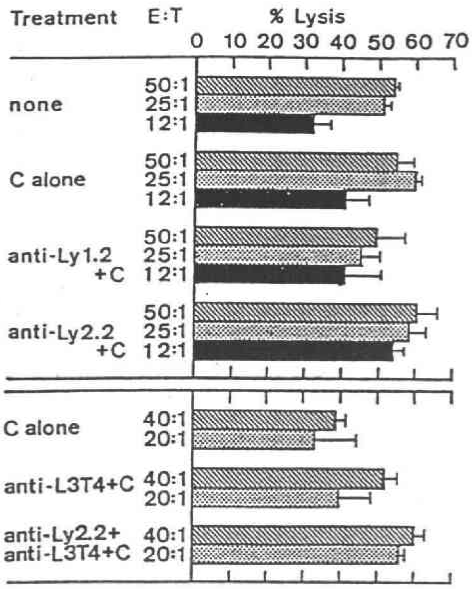

Fig. 2–7. Negative selection of LAK cell precursors. Hydrocortisone–resistant thymocytes were treated with alloantibody plus complement (C), and the living cells (5 x 10^6) were cultured in the presence of rh–IL–2 at 2,000 JU/ml for 5 days. The cultured cells were assayed for cytotoxicity against BMC6A tumor cells by the ^{3}H–uridine method.

Lyt 2^- HCRT (from which Lyt 2^+ cells had been eliminated by antibody plus complement) with rh–IL–2 allowed the appearance of T cells possessing high intensity of Lyt 2 antigen (Fig. 2–8). These results indicate that LAK precursor cells in thymocytes are Lyt 1^- (or dull Lyt 1^+), Lyt 2^-, L3T4$^-$, hydrocortisone–resistant cells.

DISCUSSION

"Lymphokine–activated killer" (or LAK) is a general term for IL–2–induced killer cells which show a broad spectrum of cytotoxicity against tumor cells. Therefore, it appears to involve multiple

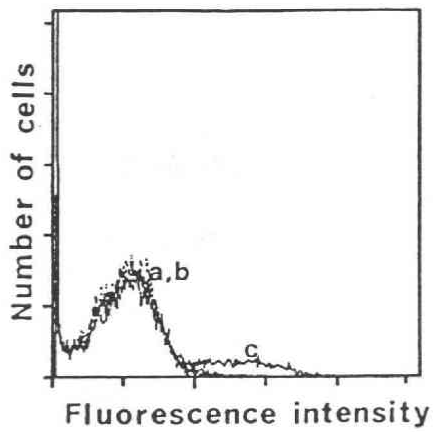

Fig. 2-8. Appearance of Lyt 2^+ cells from Lyt 2^- hydrocortisone-resistant thymocytes (HCRT) by IL-2 treatment. HCRT were treated with anti-Lyt 2 antibody plus complement twice. The remaining living cells were then stained with FITC-conjugated anti-Lyt 2 antibody before (a) or after culture with rh-IL-2 for 3 days (b) or 5 days (c) and their fluorescence intensity was determined by flow cytometry.

cell populations. In the mouse system, for example, LAK cells from spleen contain at least two cell types; one is assigned as asialoGM_1^+ LAK of NK cell type and the other as Thy 1 rich, Lyt 2^+, asialoGM_1^- LAK of T cell type (21). Moreover, LAK cells from athymic nude mouse express Thy 1 but not Lyt 2 alloantigen (26).

The results reported in this paper demonstrated that stimulation of thymocytes with IL 2 also allowed the induction of LAK cells, if the mice had been pretreated with HC, and that the induced LAK cells expressed Lyt 2 and high density LAA, but not Lyt 1 alloantigen on their cell surface. With regard to the LAK precursor, it is probable that the precursor is immature thymocytes. It is known that there are two types of immature thymocyte populations, an HC-sensitive Lyt 2^+, L3T4$^+$ population and an HC-resistant Lyt 2^-, L3T4$^-$ population, and the latter population is found to respond proliferatively to IL-2 (33-36). Indeed, negative selection assay using antibody and complement and the following phenotype assay by flow cytometry revealed that the LAK precursor cells are deficient in Lyt 2 antigen and that the IL-2 treatment allowed the emergence of this antigen in the induced

LAK cells. As to Lyt 1, negative selection with anti-Lyt 1 antibody plus complement eliminated the bright Lyt 1^+ cell population. Since these treatments did not affect the induction of LAK cells, it is indicated that the precursor cells in thymus are Lyt 1^- or dull Lyt 1^+, L3T4$^-$, Lyt 2^- T cells.

As judged from the previous and the present results, all mouse LAK cell populations, irrespective of the cell source, strongly express both Thy 1 and LAA (LFA-1) antigens on their cell surface, and they exhibit similarly broad and strong cytotoxicity against a variety of mouse tumor cells. However, the expressions of Lyt antigens and asialoGM_1 differ depending on the source of the cells. It is generally accepted that binding of killer cells to the target is the first obligatory step for target cell destruction. I observed that LAK cells also bind to target tumor cells in the initial step of killing (10). I also investigated the molecule responsible for the cell-to-cell binding and the subsequent cytocidal reaction. Addition of anti-LAA monoclonal antibody (KBA), but not of anti-Thy 1 antibody, blocked the LAK cell binding to target tumor cells and the destruction of target cells (10). Since LAA antigen is commonly expressed on the LAK cell surface, LAA is considered to be a candidate for a molecule which controls the target cell-binding of the LAK cells.

III. The Role of Lymphokine-Activated Cell-Associated Antigen: Distribution and Correlation with Cell Cycle

INTRODUCTION

The investigation of the role of cell surface molecules involved in the recognition and the activation of lymphocytes is an important issue for the evaluation of the mechanisms of immune regulation.

I previously reported that KBA monoclonal antibody (mAb), derived from hybridoma cells established by fusing the mouse myeloma P3X63AG8.653 with spleen cells of SD rat immunized with mouse lymphokine-activated killer (LAK) cells, was highly reactive to LAK cells and could block the cytotoxicity of broad-reactive killer (BRK) cells, including LAK cells, natural killer (NK) cells, and activated macrophages (A-Mϕ), but not cytotoxic T lymphocytes (CTL) (10). KBA mAb recognizes molecules that consist of two distinct polypeptides with mw 180kDa and 95kDa. I designated this antigen lymphokine-activated cell-associated antigen (LAA). The MW of LAA was similar to that of LFA-1, but KBA mAb appeared to recognize some different epitopes from those defined by the M17/4 mAb against LFA-1, because the reactivity of KBA mAb with CTL and bone marrow cells was different from that of M17/4 mAb against LFA-1 (15, 37).

LAA is distributed on all lymphocytes, but the amount of LAA on these unstimulated lymphocytes is low. Stimulation of spleen cells with recombinant interleukin 2 (r-IL-2) resulted in a remarkable increase of the level of LAA. This fact strongly suggested that LAA molecules were closely related to the activation of lymphocytes.

In this paper, I investigate the LAA expression on in vitro and in vivo activated lymphocytes and tumor cell lines and demonstrate the correlation of LAA expression with the cell cycle of activated T cells.

RESULTS

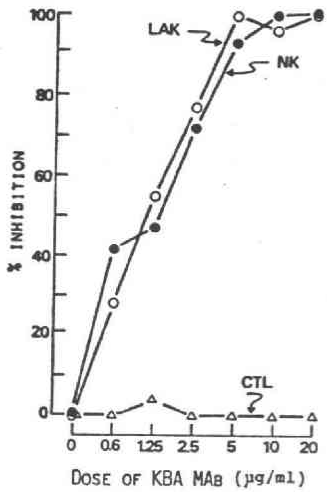

Fig. 3–1. Effect of KBA mAb on the cytotoxic activity of LAK cells, NK cells, and CTL. LAK cells (○), NK cells(●), and CTL(△) were obtained from C57BL/6 mouse spleen cells or PEC as described under Materials and Methods. These killer cells cocultured for 18 h at 37°C with [^{3}H]uridine–labeled target cells in the presence or absence of various concentrations of KBA mAb. MBL–2, YAC–1, and RADA1 lymphomas were used as target cells for LAK, NK, or CTL, respectively. Effector to target ratio was 50:1.

Blocking of broad–reactive killer–mediated cytotoxicity by purified KBA mAb. As previously reported (10), the addition of culture supernatant containing KBA mAb to the LAK–mediated cytotoxicity assay resulted in the complete inhibition of LAK activity. Moreover, it was demonstrated that KBA mAb could also block the cytotoxicity of NK cells, but not CTL. In the present experiments, I examined the effect of KBA mAb on the cytotoxic activity of LAK cells, NK cells, and CTL by using purified KBA mAb to exclude trivial inhibitory mechanisms such as the nonspecific toxic effect of the culture supernatant by hybridoma cells. The results are illustrated in Fig. 3–1. In agreement with our previous results, purified KBA mAb strongly inhibited the cytotoxic activity of

both LAK cells and NK cells, but not CTL.

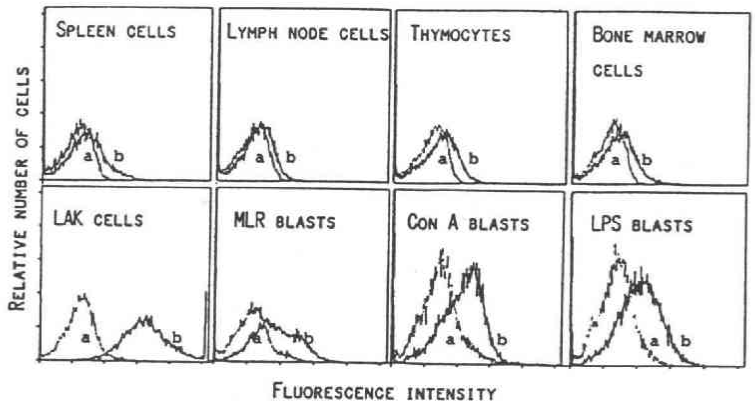

Fig. 3–2. The expression of LAA on resting and *in vitro* activated lymphocytes. LAA expression on unstimulated lymphocytes (spleen cells, lymph node cells, thymocytes, bone marrow cells) or *in vitro* activated cells (LAK, MLR–blasts, Con A–blasts, and LPS–blasts) was measured using biotinylated KBA mAb, FITC–avidin, and FACS analyzer. The staining procedure and preparation of activated cells are described under Materials and Methods. (a) Control curve of cells stained with avidin FITC alone; (b) LAA expression on the cells.

Expression of LAA on activated lymphocytes. Normal spleen cells, lymph node cells, thymocytes, and bone marrow cells all expressed low levels of LAA. In contrast, activation of spleen cells with either Con A, LPS, or allo–antigens resulted in a dramatic increase in LAA expression. The largest increase of LAA expression was observed when spleen cells were cultured with r–IL–2 (Fig. 3–2).

I also examined LAA expression on in vivo activated lymphocytes (Fig. 3–3). A Streptococcus preparation designated OK–432, was used as a in vivo stimulant. As shown in Fig. 3–3, nylon passed normal PEC expressed low levels of LAA, whereas nylon passed OK–432–activated PEC possessed high levels of LAA. Interestingly, lymphocytes infiltrating into the solid tumor, BMC2 also expressed high amounts of LAA compared with tumor–bearing mouse spleen cells.

The expression of LAA on tumor cell lines. The LAA expression of in vitro cultured tumor cell lines was investigated. All of the T–lymphoma cells tested (MBL–2, EL4, RADA1, and BW5147) expressed high amounts of LAA. The Mϕ cell line P388D_1 was also reactive with KBA

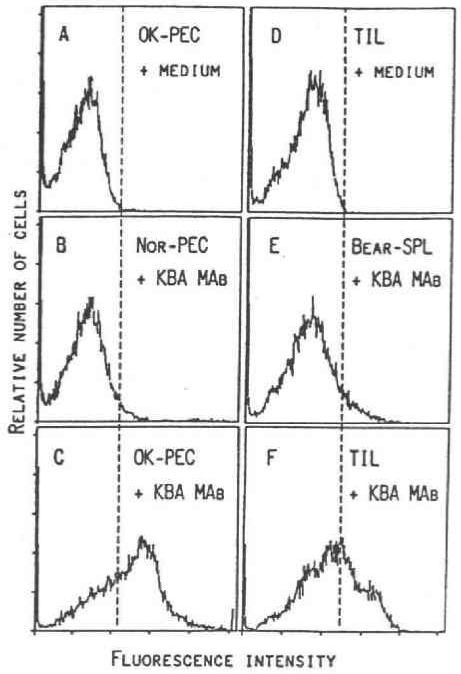

Fig. 3-3. The increased LAA expression on *in vivo* activated lymphocytes. OK-432-activated nylon-non-adherent lymphocytes (OK-PEC) and tumor-infiltrating lymphocytes (TIL) from BMC2 solid tumor were obtained as described under Materials and Methods. The LAA expression on these cells was determined using biotinylated KBA mAb, avidin FITC, and FACS analyzer. As a control, nylon-nonadherent PEC from normal mouse (Nor-PEC) and tumor-bearing mouse spleen cells (Bear-spl) were used. (A) OK-PEC treated with avidin FITC , (B) Nor-PEC stained with KBA mAb and avidin FITC, (C) OK-PEC stained with KBA mAb and avidin FITC, (D) TIL treated with avidin FITC, (E) Bear-spl stained with KBA mAb and avidin FITC, (F) TIL stained with KBA mAb and avidin FITC.

mAb. In contrast, myelomas, X5563, P3X63Ag.853, and SP2/0, fibrosarcoma, Meth A-ad and BMC2, mammary tumor, MM48, melanoma B16 showed no significant expression of LAA (Fig. 3-4). Thus, the expression of LAA appeared to be restricted in T lymphomas and Mϕ cell line.

Correlation between an increased LAA expression and cell cycle position. To examine the relationship between LAA expression and cell cycle position, C57BL/6 mouse spleen cells cultured with r-IL-2 for various times were stained with both KBA mAb and propidium iodide (PI). Figure 3-5 displays the dual parameter FACS profile for LAA (x axis) and PI (y axis). Unstimulated

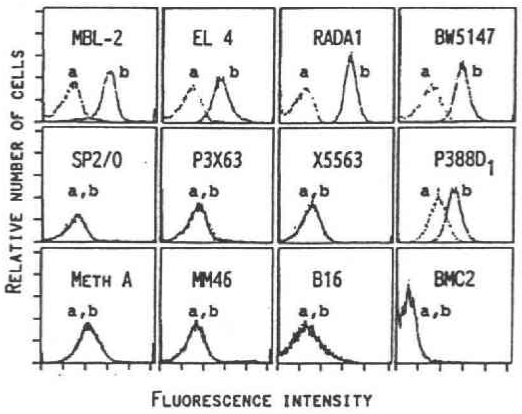

Fig. 3–4. The LAA expression on various tumor cell lines. *In vitro* maintained tumor cell lines were stained with KBA mAb and avidin FITC and their LAA expression was measured by FACS analysis. (a) Control curve of the cells treated with avidin FITC, (b) staining curve of the cells treated with biotinylated KBA mAb and avidin FITC.

lymphocytes in G_0 or G_1 phase expressed small amounts of LAA (Fig. 3–5A). However, after activation with r–IL–2 for 2 days, the expression of LAA on the cells in G_0 or G_1 phase was markedly increased. A small number of cells which had entered into S phase also expressed high amounts of LAA (Fig. 3–5B). After a 3–day culture with r–IL–2 (Fig. 3–5C), a number of cells entered into S and G_2/M phase and they expressed high levels of LAA. However, the PI method, which stains DNA but not RNA, cannot distinguish between cells in G_0 and G_1. Therefore, I next examined the effect of sodium butylate, which is known to inhibit cell transition from G_{1a} to G_{1b}, to evaluate the precise relationships between LAA expression and cell cycle. As shown in Fig. 3–6, culture of mouse spleen cells with r–IL–2 for 3 days resulted in the increased expression of LAA. In contrast, culture of spleen cells with r–IL–2 in the presence of sodium butylate resulted in the disappearance of increased LAA expression. From these results, I conclude that the increase of LAA expression is closely related to the transition phase (G_{1a} to G_{1b}) of the cell cycle.

DISCUSSION

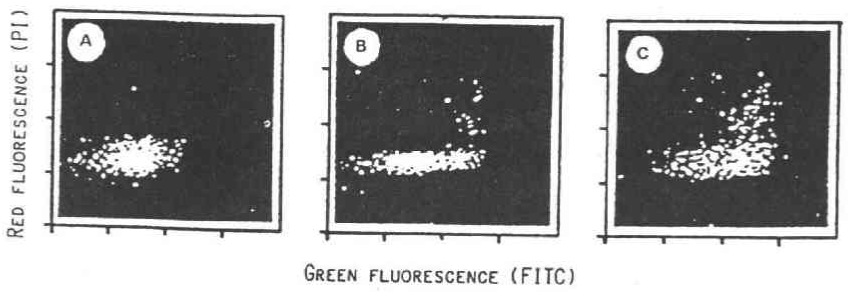

Fig. 3-5. The relationships between LAA expression and cell cycle. C57BL/6 mouse spleen cells were double-labeled with propidium iodide (PI) and biotinylated KBA mAb plus avidin FITC before or after culture with 2,000 JU/ml of r-IL-2. (A) Spleen cells before culture, (B) spleen cells cultured with r-IL-2 for 2 days, (C) spleen cells cultured with r-IL-2 for 3 days.

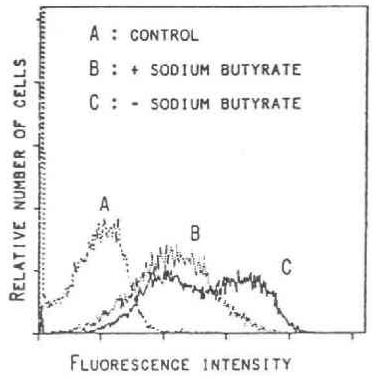

Fig. 3-6. Effect of sodium butyrate on LAA expression. C57BL/6 mouse spleen cells (5 x 10^6/ml) were cultured with r-IL-2 (2,000 JU/ml) for 3 days in the presence or absence of 1 *mM* sodium butyrate and LAA expression was assessed by using KBA mAb, avidin FITC, and FACS analyzer. (A) Control curve of IL-2-activated cells treated with avidin FITC, (B) staining curve of the cells activated with r-IL-2 in the presence of sodium butyrate, (C) staining curve of the cells cultured with r-IL-2 in the absence of sodium butyrate.

Recent reports have demonstrated that stimulation of lymphocytes with interleukin 2 results in the generation of LAK cells which are distinct from CTL and NK cells (27, 7, 8). LAK cells can lysis a variety of tumor cells in vitro and show strong anti-tumor effect in vivo (22, 24). However, the detailed mechanisms of LAK-mediated cytotoxicity remain unclear.

To evaluate this problem, I prepared KBA mAb by fusing mouse myeloma cells with spleen cells from rats which had been previously immunized with mouse LAK cells. In accordance with

previous results (10), purified KBA mAb completely blocked the LAK activity against NK-resistant MBL-2 lymphoma cells. Moreover, it was demonstrated that NK activity was highly sensitive to KBA mAb, whereas CTL activity was insensitive to KBA mAb (Fig. 3-1). From these results, I concluded that LAA, defined KBA mAb was closely related to the broad-reactive killer cell-mediated cytotoxicity.

Cell surface molecules involved in the cell-mediated cytotoxicity such as T-200, Lyt 2, and LFA-1 antigens, etc. have previously been examined by others (38, 39). LAA differs from T-200 and Lyt 2 antigen in its molecular weight. However, the character of LAA is similar to the molecular size of LFA-1, which is known to be a molecule involved in CTL-mediated cytotoxicity (40-42). Indeed, recently it was demonstrated that KBA mAb recognizes distinct epitopes of LFA-1 systems defined with M17/4 mAb against LFA-1 (our unpublished observation).

The increased expression of LFA-1 on activated lymphocytes was first reported by Davignon et al. (43). In agreement with their results, I confirmed that LAA epitopes on LFA-1 systems are increased when spleen cells are activated with either Con A, LPS, or allo-antigens. Moreover, I now demonstrate that IL-2 is the best signal for the induction of LAA (Fig. 3-2). From these results, I conclude that the increased expression of LAA by mitogens may be induced by IL-2, which is endogenously produced by helper T cells. It remains unclear whether other lymphokines, such as IL-3 and BCGF (44, 45) can induce high amounts of LAA on the cells.

The observation that in vivo activated lymphocytes, such as OK-432 activated PEC and TIL from BMC2 (Fig. 3-3) express high amounts of LAA suggests an important role of LAA epitopes on LFA-1 molecules in vivo. OK-432 activated PEC mainly consisted of Thy 1.2^+, Lyt 1^-, Lyt 2^- and asialoGM_1^+ NK cells (our unpublished data). These results suggest that LAA expression appears to be correlated with NK cell-activation in vivo.

The expression of LAA was restricted to T-cell lymphomas and Mϕ cell line with other types of tumor cells not expressing LAA (Fig. 3-4). LAA appear to be absent from myeloma cell lines in spite of the finding that high levels of LAA are expressed on LPS-activated B cells. I have no data

on B-lymphomas, and it thus remains unclear whether B-lymphoma cells express LAA.

The increased expression of LAA was closely related to the transition phase (G_{1a} to G_{1b}) of the cell cycle, indicating that LAA is a cell surface molecule important in the early stages of T-cell activation (Figs. 3-5 and 3-6). The precise role of LAA in T-cell activation will be described in the next chapter.

IV. The Role of Lymphokine-Activated Cell-Associated Antigen: Inhibition of T Cell Activation by a Monoclonal Killer-Blocking Antibody

INTRODUCTION

Triggering of T cells via T-cell antigen-receptor (46-48) is one of the earliest step in the activation of T cells. However, the development of monoclonal antibodies (mAb) to the cell surface molecules on immunoregulatory cells has suggested that several additional membrane proteins on T cells or antigen-presenting cells (APC) are required for the early stages of T-cell activation. MHC molecules, Lyt 2, L3T4, and LFA-1 are all well known functional T-cell surface antigens (11, 37, 49, 50). However, the precise role of these accessory molecules is still unclear.

I recently established hybridoma cells producing KBA mAb which is able to block the cytotoxicity of LAK cells (10). This mAb recognizes molecules similar to LFA-1 consisting of two glycoprotein subunits (mw 180kDa and 95kDa) (40, 51, 52). However, KBA mAb seems to recognize different epitopes from the LFA-1 antigen epitope defined by M17/4 mAb. Therefore, I designated this antigen lymphokine-activated cell-associated antigen (LAA). As described in Chapter III, both in vitro and in vivo activated lymphocytes express high amounts of LAA. Furthermore, the increased expression of LAA is associated with the cell cycle phase of the activated T cells. These data encouraged us to investigate the role of LAA in T-cell activation.

In this paper, I demonstrate that LAA plays an important role in T-cell response to concanavalin A (Con A), class I antigen, and class II antigen in the early stages of T-cell activation, such as the phase of IL-2 receptor acquisition and IL-2 production. However, LAA does not appear to be important in T-cell response to phytohemagglutinin (PHA) and IL-2.

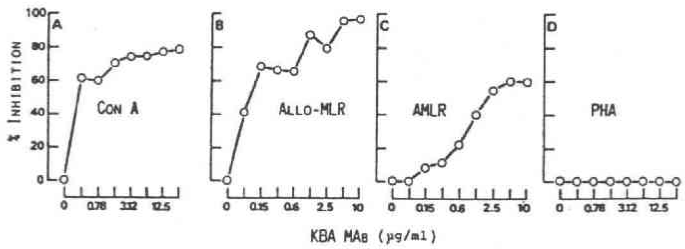

Fig 4–1. Inhibition of T–cell responses by KBA mAb. C57BL/6 mouse spleen cells (5 x 10^6) were cultured with either Con A (A), allogeneic spleen cells (B), autologous spleen cells (C), or PHA (D) in the presence or absence of KBA mAb as described under Materials and Methods. The inhibitory effect of KBA mAb was shown as a inhibition percentage, which was calculated by the following formula: % inhibition = (1 – cpm of spleen cells cultured with KBA mAb / cpm of spleen cells cultured without KBA mAb) x 100.

Effect of KBA mAb on various immune responses. To examine the role of LAA in T–cell responses, I examined the effect of KBA mAb on a variety of in vitro immune responses. The results are illustrated in Fig. 4–1. The addition of KBA mAb caused a significant inhibition of T–cell responses to Con A, class I antigen, and class II antigen. In striking contrast, T–cell responses to PHA were not blocked by KBA mAb addition. In all these immune responses, addition of rat mAb against mouse Thy 1.2 antigen showed no inhibitory effect (data not shown). These data show that the inhibitory effect of KBA mAb on immune responses is specific.

LAA is required for the early stages of T–cell activation. The experiment illustrated in Fig. 4–2 was designed to clarify the stage of T–cell activation in which LAA is required. As shown in Fig. 4–1, simultaneous addition of KBA mAb in Con A responses resulted in a significant inhibition of T–cell proliferation. Strong inhibitory effects of KBA mAb were also observed when KBA was added to the culture at early times (0, 1, 2, 4 h) after Con A stimulation. However, the inhibitory effect of KBA mAb declined when it was added to Con A response later in the culture period (6, 8,

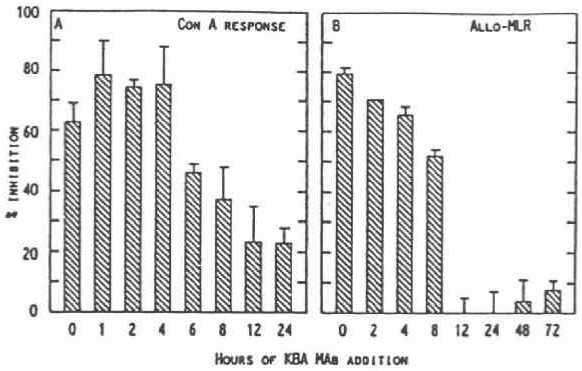

Fig. 4–2. KBA mAb inhibits the early stage of T–cell activation. (A) C57BL/6 mouse spleen cells (5 x 10^6/ml) were cultured with 2.5 μg/ml of Con A for 32 h and pulsed with [^{3}H]TdR for 4 h. KBA mAb was added to this culture at various times after Con A stimulation. (B) C57BL/6 mouse spleen cells (5 x 10^6/ml) were cocultured with MMC–treated A/J spleen cells (5 x 10^6/ml) for 96 h, and pulsed with [^{3}H]TdR after replacing culture medium with fresh medium. KBA mAb was added to this culture at various times after stimulation.

12, 24 h). The same results were obtained in Allo–MLR assays. Addition of KBA mAb early in the culture (0, 2, 4, 8 h), but not at later times (12, 24, 48, 72 h), resulted in strong inhibition. These data indicate that LAA plays an important role in T–cell activation at the early stages.

Blocking of the acquisition of IL–2 receptors by KBA mAb. C57BL/6 mouse spleen cells were cultured with or without Con A or PHA for 8 h at 37°C, and passed through nylon wool columns after removing cell–bound Con A or PHA by washing with 0.1 *M* α–methyl–D–mannoside or *N*–acetyl–D–galactosamine. The responsiveness of T cells to r–IL–2 (2000 JU/ml) was then measured. As shown in Fig. 4–3, cells preincubated without Con A showed no significant [^{3}H]TdR incorporation regardless of the presence or absence of r–IL–2. In contrast, the cells pretreated with Con A revealed strong proliferative responses in the presence of r–IL–2, but not in the absence of r–IL–2. These data indicated that an 8–h Con A pulse was sufficient to induce T–cell IL–2 receptors. As shown in Fig. 4–3, addition of KBA mAb in the preculture period blocked the acquisition of T–cell IL–2 responsiveness (indicative of the expression of IL–2 receptors) in response to Con A. In contrast to this result, the acquisition by T cells of IL–2 receptors by activation with PHA was not blocked by

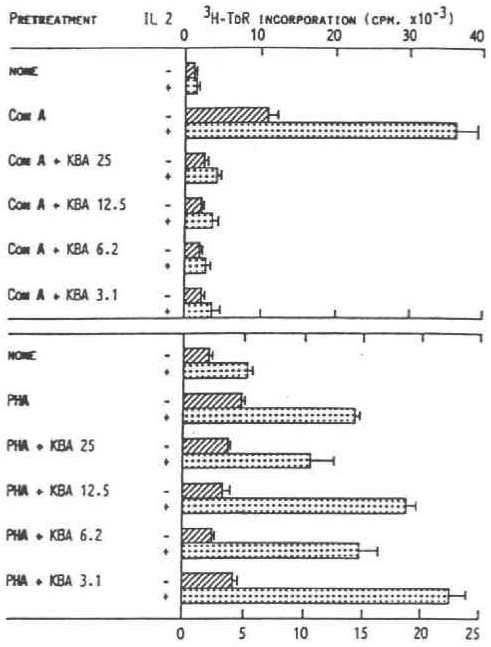

Fig. 4–3. Effect of KBA mAb on the acquisition of IL–2 receptors. C57BL/6 mouse spleen cells (5 x 10^6/ml) were stimulated with Con A (5 μg/ml) or PHA (20 μg/ml) for 8 h in the presence or absence of various concentrations of KBA mAb (25, 12.5, 6.25, 3.125 μg/ml). After washing with 0.1 *M* α–methyl–D–mannoside or 0.1 *M N*–acetyl–D–galactosamine to remove cell bound Con A or PHA, the cells were passed through nylon wool columns and cultured with or without r–IL 2 (2,000 JU/ml) for 32 h. After culture, the cells were pulsed with [^{3}H]TdR for 4 h.

the addition of KBA mAb.

Effect of KBA mAb on IL–2 production and IL–2 action. I next examined the effect of KBA mAb on IL–2 production. The IL–2 activity was determined using the IL–2–dependent T–cell clone T572. When T572 cells were cultured with culture supernatant of Con A–stimulated spleen cells, they showed a dose–dependent response to IL–2. However, in the presence of KBA mAb, culture supernatant of Con A–stimulated spleen cells showed greatly reduced IL–2 activity. To remove the possibility that the inhibitory effect of KBA mAb on IL–2 production was derived from the KBA mAb carried into the Con A–sup, the effect of KBA mAb on IL–2 action was determined. As shown in

Table 4-1

Effect of KBA mAb on the action of IL-2[a]

Dose of KBA mAb (μg/ml)	[^{3}H]TdR incorporation (cpm)	% Inhibition
0.0	57,723 ± 1939	
0.6	55,379 ± 191	0.4
1.2	52,463 ± 238	0.9
2.5	60,430 ± 1234	-0.4
5.0	58,192 ± 4665	-0.1
10.0	60,625 ± 5709	-0.5
20.0	72,150 ± 748	-24.0

a) **IL-2-dependent T-cell clone, T572 cells (10^4), were cultured with 20 JU/ml of r-IL-2 in the presence or absence of various concentrations of KBA mAb. After culture for 32 h, the cells were pulsed with [^{3}H]TdR for 4h.**

Fig. 4-4, the proliferative responses of T572 cells were not inhibited by addition of KBA mAb to the IL-2 assay. The inability of KBA mAb to block the action of IL-2 was confirmed using r-IL-2. As shown in Table 4-1, KBA mAb showed no significant inhibitory activity on the r-IL-2-dependent proliferation of T572 cells in any of the doses used. The overcoming effect of IL-2 was not attributable to the failure of KBA mAb to inhibit IL-2 action, because KBA mAb could not inhibit the proliferation of T572 cells even in the presence of suboptimal doses of r-IL-2 (less than 20 JU/ml) (Fig. 4-5). The inhibitory effect of KBA mAb on IL-2 production is strong, and less than 1 μg/ml of KBA mAb is enough to block IL-2 production (Table 4-2).

Table 4-2

Effect of KBA mAb on the production of IL-2[a]

Dose of KBA mAb (μg/ml)	IL-2 activity (cpm)	% Inhibition
0.0	10,518 ± 295	
0.6	1,992 ± 651	74.8
1.2	3,375 ± 172	67.9
2.5	3,358 ± 502	68.1
5.0	2,634 ± 436	74.9
10.0	2,301 ± 473	78.1
20.0	1,632 ± 21	84.4

a) C57BL/6 mouse spleen cells (5 x 10^6/ml) were cultured with Con A (5 μg/ml) in the presence or absence of KBA mAb for 20 h at 37°C. After incubation, the culture supernatants were harvested and their IL-2 activity was measured by using T572 cells as described under Materials and Methods.

DISCUSSION

In this paper, I demonstrated that LAA, defined by KBA mAb, plays an important role not only in broad-reactive killer cell-mediated cytotoxicity but also in various kinds of T-cell responses.

The process of lymphocyte activation is accompanied by the acquisition of various kinds of cell surface molecules, including T-cell receptors, MHC molecules, and IL-2 receptors (46-48, 53, 54). Furthermore, a recent work indicates that LFA-1 and L3T4 molecules play a noteworthy role in T-cell activation (37, 55). However, the precise role of these accessory molecules remains unclear.

To clarify the role of LAA, which is one of the epitopes of LFA-1 systems (our unpublished

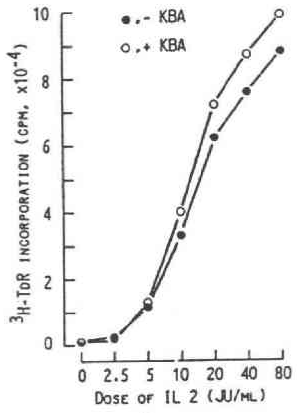

Fig. 4–4. Effect of KBA mAb on the action of IL–2. IL–2–dependent T–cell clone, T572 cells (10^4), were cultured with various concentrations of r–IL–2 in the presence (○) or absence (●) of 20 μg/ml of KBA mAb for 32 h and then pulsed with [^{3}H]TdR for 4 h.

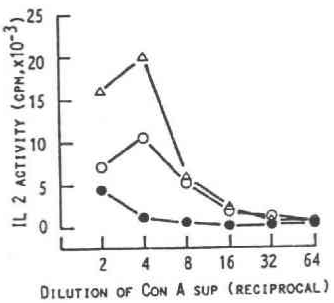

Fig. 4–5. Effect of KBA mAb on the production of IL–2. C57BL/6 mouse spleen cells (5 x 10^6/ml) were cultured with 5 μg/ml of Con A in the presence (●) or absence (○) of KBA mAb (20 μg/ml) for 20 h. After incubation, the culture supernatants (Con A–sup) were harvested and their IL–2 activity was measured using T572 cells as described under Materials and Methods. The IL–2 activity of Con A–sup with 20 μg/ml of KBA mAb added was also determined (△).

observation), in T–cell activation, I investigated the effect of KBA mAb on various immune responses and demonstrated the following results: 1) KBA mAb can block T–cell responses to Con A, class I, and class II, but not to PHA (Fig. 4–1); 2) LAA, defined by KBA mAb, plays an important role in T–cell activation at the early stages (Fig. 4–2); 3) Con A–induced, but not PHA–induced, IL–2

receptor acquisition is blocked by KBA mAb (Fig. 4–3); 4) IL–2 production of Con A–activated spleen cells is inhibited by KBA mAb (Fig. 4–4 and Table 4–2); 5) The action of IL–2 on a T–cell line is not inhibited by KBA mAb (Fig. 4–5 and Table 4–1).

The precise role of LAA epitopes of LFA–1 molecules remains unclear. However, recent results indicate that LFA–1 molecules are essential for cell–cell contact of lymphocytes (56). Therefore, blocking of T–T or T–Mϕ interaction by KBA mAb may be a major cause of the reduction of T–cell responses to a variety of antigens. Interestingly, T–cell response to PHA is not inhibited by KBA mAb. This result indicates that mouse T–cell activation might be composed of two distinct activation pathways in association with LAA molecule dependency.

I am currently investigating the biochemical and functional difference between LAA epitopes defined by KBA mAb and the LFA–1 epitopes recognized by M17/4 mAb.

V. Immune Deficiency of the CTS Mouse: Deficiency of in vitro T Cell-Mediated Immune Response

INTRODUCTION

The cataract Shionogi (CTS) mouse was originally derived from outbred ICR mice. Starting from one mouse with cataract and small eyes found in a closed colony of ICR mice, an inbred CTS strain was established at Shionogi Aburahi Laboratories (57, 58). In view of clinical trend that cataracts are often observed in diabetic patients, two sublines have been separated during the course of breeding of the CTS mouse with monitoring blood glucose levels, one being the non-obese diabetic (NOD) strain and the other the non-obese-non diabetic (NON) strain. The NOD mouse has widely been used as a good model of human type I diabetes mellitus. This mouse spontaneously develops insulitis, and insulin-dependent diabetes mellitus. Approximately 80% of the females and 20% of the males develop overt diabetes by 30 weeks of age (59). Breeding studies by Makino et al. (60) and others (61) have elucidated that at least two autosomal recessive genes determine the diabetes susceptibility, one being linked to major histocompatibility complex (MHC) (particularly to class II MHC) on chromosome 17 (62, 63) and the other being located on chromosome 9 (64). Recent investigation suggests that CTS and NON strains carry one of these two genes. The CTS mouse has the same unique class II MHC as that of the NOD mouse which can be characterized by the presence of the peculiar I-A_β and the absence of I-E, though different in the K and D regions of class I MHC (65). Therefore, the CTS mouse very likely possesses the class II-correlated diabetic gene. On the other hand, breeding studies in crosses of NOD with NON mice revealed that the development of insulitis is regulated by a single recessive gene (66, 67) in contrast with the two-gene regulation in crosses of NOD with C57BL/6J mice (60). Because the MHC haplotype of the NON mouse (H-2 $K^bI\text{-}A^{(non)}D^b$) is different from that of the NOD mouse (H-2 $K^dI\text{-}A^{(nod)}D^b$) (65), it is likely that the diabetogenic gene

which the NON mouse carries is the MHC-non-linked one. In view of these findings, analysis of the biological characteristics of CTS and NON strains might contribute something to gain an insight into the pathogenic mechanism of the NOD mouse. In addition, the biological characterization of these strains might show up their own usefulness for the research in the other field than the diabetes, independently of the NOD mouse. In the meantime, with an aim of ascertaining that each strain is genetically uniform and independent to each other, I performed reciprocal skin graft tests among the NOD, CTS and NON strains, and found that the CTS mouse failed to reject the graft from the NOD mouse in contrast with the rejection of the CTS graft in the NOD mouse (67). This was an unexpected result, because these two strains are different in both H-2K and H-2D regions: the H-2K molecules of the CTS mouse are capable of reacting with anti-H-$2K^k$ as well as anti-H-$2K^d$ monoclonal antibodies and the H-2D molecules in capable of reacting with any one of the anti-H-$2D^b$, anti-H-$2D^d$, anti-H-$2D^k$ and anti-H-$2D^q$ monoclonal antibodies (65). The failure of the CTS mouse to reject the NOD mouse skin graft irrespective of the difference in class I MHC suggests that the CTS mouse is impaired in immune responsiveness.

On such a background, I studied the immune responses in the CTS mouse. The present study is concerned with several in vitro immune responses and flow cytometric analysis of the lymphocyte populations in the spleen.

RESULTS

Response of the CTS spleen cells to T-cell and B-cell mitogens. Table 5-1 summarizes the responsiveness of splenocytes from the CTS, NOD, NON, and control mice to T and B cell mitogens. The proliferative responses of splenocytes from NOD and NON mice to various mitogens were nearly

Table 5-1

Mitogen response of spleen cells of the CTS and control mice

Mitogen	Dose (μg/ml)	^{3}H-TdR incorporation (cpm)				
		NON	NOD	CTS	C3H/He	C57BL/6
Con A	2.0	100691 ± 6882[a] (22.5)	93556 ± 3570 (20.6)	15447 ± 651 (9.1)	87062 ± 12205 (21.1)	76503 ± 22338 (29.3)
	1.0	44862 ± 4719 (10.0)	76133 ± 1777 (16.8)	5400 ± 203 (3.2)	70915 ± 2713 (17.2)	78960 ± 8366 (33.7)
PHA	4.0	19533 ± 2207 (4.4)	28414 ± 1473 (6.3)	4107 ± 332 (2.4)	35065 ± 17532 (8.5)	26815 ± 15012 (10.5)
	2.0	13956 ± 2335 (3.1)	18230 ± 436 (4.0)	2540 ± 87 (1.5)	17399 ± 6565 (4.2)	39390 ± 639 (15.1)
LPS	2.0	62489 ± 5914 (14.0)	53327 ± 3450 (11.8)	48411 ± 810 (28.4)	49993 ± 24997 (12.1)	73063 ± 6314 (28.0)
	0.5	63594 ± 1404 (14.2)	57566 ± 1259 (12.7)	50314 ± 3041 (29.5)	57859 ± 1291 (14.0)	50703 ± 2579 (19.4)
None		4476 ± 135	4533 ± 148	1703 ± 111	4123 ± 266	2612 ± 183

Spleen cells were cultured with Con A, PHA or LPS for 48 h at 37°C. After culture, the cells were pulsed with [^{3}H]TdR for 4 h.

a) Mean ± SE of triplicate samples
(Stimulation index)

the same as those of splenocytes from C3H/He and C57BL/6 mice. In contrast, the splenocytes from the CTS mice were very low in responsiveness to T cell mitogens such as Con A and PHA but not to the B cell mitogen, LPS.

Response of the CTS spleen cell to alloantigens. In mixed-lymphocyte culture assays (Table 5-2), C3H/He splenocytes proliferated when cocultured with splenocytes of the NOD or CTS mice. However, the splenocytes of the CTS mice did not proliferate in general, when cocultured with splenocytes of various strains. With NOD splenocytes, the failure of proliferative response was

Table 5-2

Mixed lymphocyte reaction of the splenic lymphocytes of CTS and the other mice

Responder	Stimulator	^{3}H-TdR incorporation (cpm)			
		1:1[a]	2:1	4:1	-
Expt.1					
CTS	NOD	18561 ± 1179[b] (2.1)	17042 ± 1027 (1.9)	12457 ± 1407 (1.4)	8921 ± 235
	C3H/He	21892 ± 992 (2.5)	21055 ± 2565 (2.4)	18441 ± 1384 (2.1)	
NOD	CTS	30684 ± 1820 (1.3)	28089 ± 3164 (1.2)	14701 ± 473 (0.6)	23665 ± 160
	C3H/He	62137 ± 6982 (2.6)	88280 ± 4321 (3.7)	80212 ± 2335 (3.4)	
C3H/He	CTS	24825 ± 2022 (6.7)	22287 ± 6053 (6.0)	10716 ± 3192 (2.9)	3720 ± 1426
	NOD	49993 ± 4034 (13.4)	40934 ± 7841 (11.0)	18745 ± 3145 (5.0)	
Expt. 2					
CTS	CBA/N	10945 ± 936 (0.7)	14333 ± 3085 (1.0)	9759 ± 1208 (0.7)	14792 ± 986
	C3H/He	14144 ± 1026 (1.0)	14890 ± 2634 (1.0)	11192 ± 499 (0.8)	
	C57BL/6	20701 ± 1295 (1.4)	16381 ± 2438 (1.1)	19617 ± 2193 (1.3)	
	DS	20722 ± 1969 (1.4)	15384 ± 1540 (1.0)	14487 ± 1632 (1.0)	
C3H/He	C57BL/6	46667 ± 6861 (5.2)	30565 ± 768 (3.4)	19600 ± 1968 (2.2)	8936 ± 419

Responder spleen cells were cocultured with MMC-treated stimulator spleen cells for 96 h, and pulsed with [^{3}H]TdR after replacing the culture medium with fresh medium.

a) Responder-to-stimulator ratio

b) Mean ± SE of triplicate samples
(Stimulation index)

observed only when they were cocultured with splenocytes of the CTS mice. In primary mixed

Table 5-3

CTL generation from the splenic lymphocytes of the CTS, NOD and C3H/He mice

Responder	Stimulator	Percent cytotoxicity		
		50:1[a]	25:1	12.5:1
CTS	NOD	1.1 ± 0.6[b]	0.0 ± 0.0	0.0 ± 0.0
	C3H/He	16.3 ± 0.5	10.5 ± 1.0	5.8 ± 0.8
	CTS	1.1 ± 0.8	NT[c]	NT
NOD	NOD	1.1 ± 0.5	NT	NT
	C3H/He	59.0 ± 2.0	40.7 ± 5.3	29.9 ± 1.6
	CTS	55.8 ± 1.2	41.6 ± 2.4	31.7 ± 5.6
C3H/He	C3H/He	7.7 ± 1.1	NT	NT
	CTS	66.9 ± 2.9	69.9 ± 1.9	62.2 ± 5.6

Responder spleen cells (5 x 10^6/ml) were cocultured with MMC-treated stimulator spleen cells (2.5 x 10^6/ml) for 96 h at 37°C. Cytotoxic activity of the spleen cells was measured as described under Materials and Methods.

a) Effector-to-target cell ratio

b) Mean ± SD of triplicate samples

c) Not tested

lymphocyte culture, the generation of CTL from the splenocytes of the CTS mouse was less than that from the splenocytes of the control strains (Table 5-3). These results suggest that the CTS mouse is deficient in T lymphocytes.

IL-2 production of spleen cells in the CTS mouse. As the responsiveness to T cell mitogens in the CTS mouse was lower than that in the control strains, I next studied the production of IL-2 by Con A-stimulated splenocytes of the CTS mouse. The IL-2 activity was determined using the IL-2-

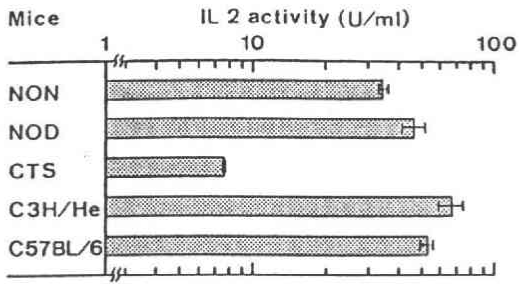

Fig. 5–1. Production of IL 2 by Con A–stimulated lymphocytes of the CTS and other mice at 10 weeks of age. Spleen cells (2.5 x 10^6/ml) were cultured with 5 μg/ml of Con A for 20 h. Then, the culture supernatants were harvested and their IL 2 activity was assayed using CTLL–2 cells, as described in Materials and Methods.

dependent T cell clone CTLL–2. The IL–2 activity of Con A–sup from reference strains was very high, ranging from 30 to 70 units/ml. However, Con A–sup obtained from CTS mouse splenocytes had much lower IL–2 activity, 7 units/ml (Fig. 5–1).

IL–2 receptor expression of CTS spleen cells. I next examined the Con A–induced acquisition of IL–2 receptors on CTS mouse splenocytes by means of two methods. One was the development of T cell proliferation in response to IL–2 after short–time pulsing with Con A, and the other was flow cytometric analysis using anti–IL–2 receptor monoclonal antibody, 3C7 (68). In the first method, mouse spleen cells were cultured with or without Con A for 8 h at 37°C and passed through nylon wool columns to enrich T cells after cell–bound Con A had been removed by washing with 0.1 *M* α–methyl–D–mannoside. Then the responsiveness of T cells to r–IL–2 (200 or 100 JU/ml) was measured. As shown in Figure 5–2, cells preincubated without Con A showed no significant [^{3}H]TdR incorporation regardless of the presence or absence of r–IL–2. In contrast, when pretreated with Con A, strong proliferative responses were observed in the presence of r–IL–2 but not in the absence of r–IL–2 as far as the splenocytes of C3H/He and NOD mice were concerned. These data indicated that

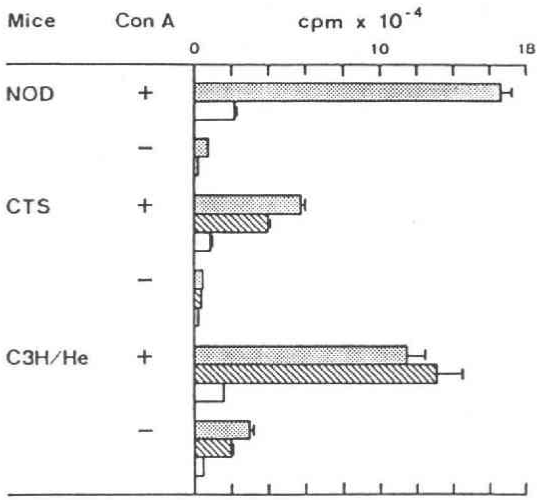

Fig. 5–2. Acquisition of IL 2 receptors by short-time Con A–stimulated lymphocytes of the CTS, NOD and C3H/He mice. Spleen cells (2.5 x 10^6/ml) were stimulated with or without Con A (5 μg/ml) for 8 h. After washing with α–methyl–D–mannoside to remove cell bound Con A, the cells were passed through nylon wool columns and these cells (5 x 105/0.2 ml/well) were cultured with r–IL 2 (200 JU/ml, ▒; 100 JU/ml, ▒; 0 JU/ml, □) for 32 h. Then, the cells were pulsed with [^{3}H]TdR for 4 h.

an 8–h Con A pulse was sufficient to induce IL–2 receptors on T cells. In the case of the CTS mouse, however, even if the cells were pretreated with Con A, they acquired only slight IL–2 responsiveness, if any, indicating little expression of IL–2 receptors. Second, by means of the flow cytometric analysis of Con A–stimulated spleen cells, it was shown that the percentages of 3C7–positive cells in the CTS mice were much lower than those in the control strains (Fig. 5–3). Thus, both the production of IL–2 and the percentage of IL–2 receptor–expressing T cells were greatly decreased in the CTS mice than in the control strains, which could explain the very low responsiveness of the CTS splenocytes to T cell mitogens.

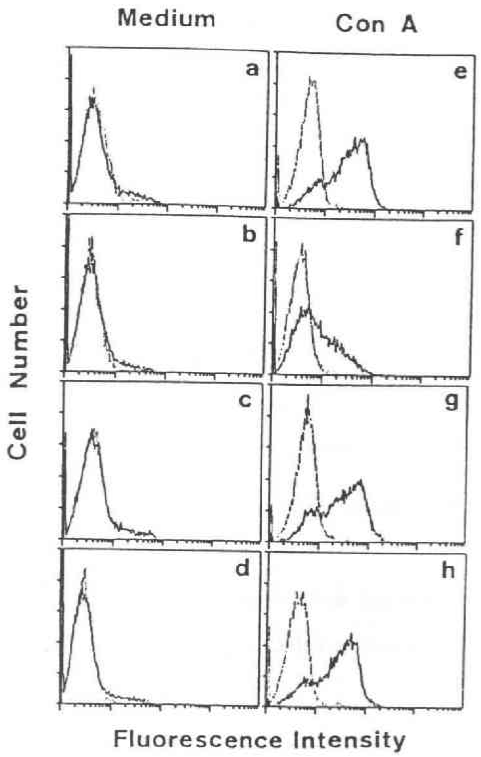

Fig. 5–3. Expression of IL 2 receptors on Con A–stimulated lymphocytes of the CTS and other mice. The spleen cells (2.5 x 10^6/ml) of NOD (a, e), CTS (b, f), C3H/He (c, g) and C57BL/6 (d, h) mice were cultured with (e–h) or without (a–d) 5 μg/ml of Con A for 24 h. After culture, the cells were harvested and incubated with (solid lines) or without (dotted lines) 10 μg/ml of FITC–labeled 3C7 monoclonal antibody at 4°C. After 30 min, cells were then washed three times and analyzed for fluorescence on a FACS 440 and a Consort 30 system (Becton Dickinson).

NK activity of the CTS splenocytes. I examined the strain difference in NK activity in mice using spleen cells from various strains (Table 5–4). The percent NK activity was highest in C3H/He and NON mice, followed by the CTS mice. The percent NK activity of BALB/c and C57BL/6 mice was lower than that of C3H/He mice. No NK activity was found with NOD mice.

Table 5–4

Strain distributions of NK activity in the spleen[a]

Mouse	NK activity	
	% of cytotoxicity[b]	Lytic $Units_{25}/10^{7}$[c]
C3H/He	25.7 ± 0.8	14.3 ± 0.2
C57BL/6	10.2 ± 0.1	0.9 ± 0.1
BALB/c	6.4 ± 0.1	0.2 ± 0.1
NOD/Shi	1.9 ± 0.6	< 0.1
CTS/Shi	14.0 ± 0.7	2.6 ± 1.4
NON/Shi	27.5 ± 1.1	14.5 ± 1.6

a) Values represent the means ± SD of three mice.

b) E/T ratio, 100/1

c) One lytic unit was defined as the number of effector cells required to cause 25% lysis of 1x 10^4 YAC–1 target cells.

Analysis of splenic lymphocyte sub–populations by flow cytometry. Lymphocyte sub–populations in spleen cells were compared in the CTS, NOD, NON and other strains using flow cytometry. Frequency of each phenotype of lymphocytes, expressed as the percentage of the cells reacting with a given antibody, is shown in Table 5–5. While Thy 1.2 antigen is expressed on T cells of NOD mice, Thy 1.1 antigen was found in the CTS and NON mice. The CTS mice showed significantly lower proportions of Thy 1.1^+, Lyt 1^+ (pan T cells), $L3T4^+$ (helper/inducer T cells), and Lyt 2^+ (suppressor/cytotoxic T cells) cells than the control strains. The deficiency of Lyt 2^+ T cells was particularly conspicuous, and accordingly, the relatively high ratio of L3T4/Lyt 2 in this strain was observed. Decrease in Lyt 2^+ cells was also observed in NON mice, although to a lesser extent than in the CTS strain.

Recovery of the response of the CTS spleen cells to T cell mitogens by enrichment of T cells.

Table 5-5

Comparison of subsets of splenic lymphocytes of CTS and the other mice

Surface markers	% of positive cells[a]					
	NON	NOD	CTS	C57BL/6	C3H/He	AKR
Thy 1.1	26 ± 3[b]	0 ± 0	9 ± 4	0 ± 0	0 ± 0	42 ± 3
Thy 1.2	0 ± 0	61 ± 5	0 ± 0	50 ± 4	33 ± 7	0 ± 0
Lyt-1	32 ± 6	67 ± 8	11 ± 2	52 ± 5	38 ± 10	52 ± 6
L3T4	20 ± 1	41 ± 3	8 ± 1	24 ± 2	19 ± 3	30 ± 2
Lyt-2	5 ± 1	20 ± 3	3 ± 1	19 ± 1	12 ± 3	14 ± 1
sIg	62 ± 2	30 ± 6	72 ± 2	42 ± 5	49 ± 5	48 ± 4
asialoGM$_1$	9 ± 3	4 ± 1	7 ± 1	7 ± 2	9 ± 2	8 ± 1
Mac-1	10 ± 2	4 ± 1	7 ± 2	7 ± 1	9 ± 1	6 ± 1
L3T4/Lyt2 ratio	4.3	2.1	3.2	1.3	1.6	2.2
Number	3	3	4	4	4	3

a) The percentages of surface marker positive cells were determined using FACS 440 and Consort 30 systems.

b) Values represent the mean and SD for the above described number of mice.

Based on the above-described results, I speculated that the decline in the number of T cells is responsible for the decreased T cell activity in the CTS mouse. To test this speculation, I studied whether the mitogen response of the CTS mouse could be restored by the enrichment of T cells. The responsiveness to PHA recovered to the levels of C3H/He and C57BL/6 mice, when splenic T cells of the CTS mouse were enriched by passing through the nylon wool column. On the other hand, the responsiveness to Con A of the CTS mouse was not recovered to the same extent as the responsiveness to PHA by the enrichment of T cells, though the Con A-responsiveness of the T cell-

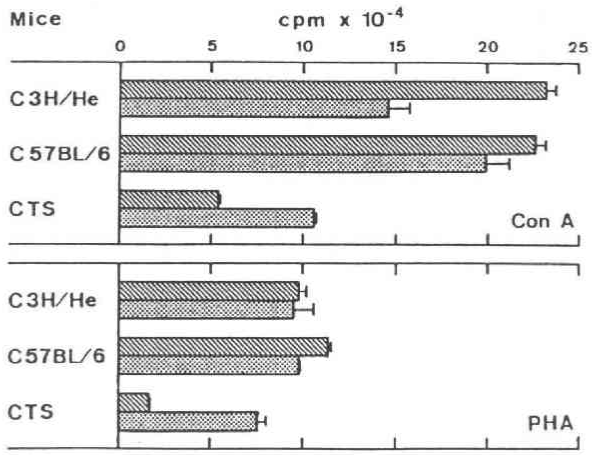

Fig. 5-4. Mitogen responses of CTS T cells passed through nylon wool columns. The spleen cells from the CTS, C3H/He and C57BL/6 mice were passed through nylon wool columns. Whole (▨) or nylon-passed (▤) cells (5 x 10^5/0.2 ml/well) were cultured with either Con A (5 μg/ml) or PHA (8 μg/ml) for 48 h. After culture, the cells were pulsed with [^{3}H]TdR for 4 h.

enriched splenocytes was higher than that of the original whole splenocyte suspension (Fig. 5-4). In addition, T cell-sorting was performed to assess the function of the purified T cells of the CTS mouse. After the spleen cells of the CTS mouse passed through the nylon wool column, the cells were co-stained with FITC-anti-Lyt 1, FITC-anti-Lyt 2, and FITC-anti-L3T4 mAb and then sorted out under sterile conditions using the FACS 440. Each suspension represented 95% of the positive sorted cells. The responsiveness to PHA of FACS-isolated CTS T cells increased to nearly the same level of the whole splenocyte of C3H/He mouse (Fig. 5-5). Therefore, the reduced T cell activity of the CTS splenocytes does not seem to be due to the dysfunction of individual T cells but to the decline in T cell number.

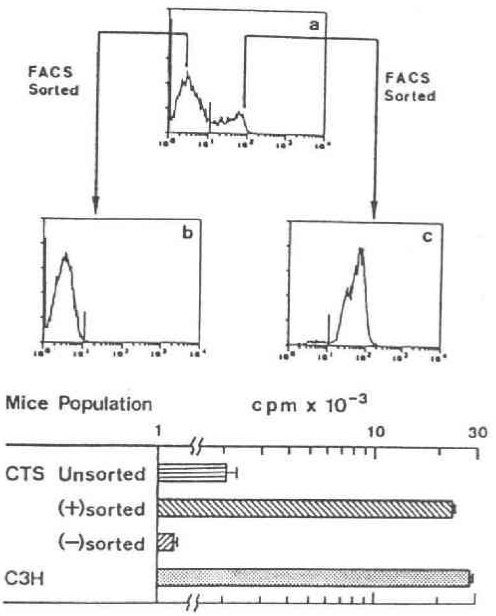

Fig. 5-5. PHA response of FACS-isolated CTS spleen T cells. After the spleen cells of the CTS mice had passed through the nylon wool column, they were co-stained with FITC-anti-Lyt-1, -anti-Lyt-2 and -anti-L3T4 monoclonal antibodies. Lyt-1+, Lyt-2+, L3T4+ T cells and Lyt-1-, Lyt-2-, L3T4- non-T cells were sorted using FACS 440 from the gates shown in (a). The purity levels of the sorted negative (b) and positive (c) cells was 99.5% and 95.5%, respectively. The populations (5 x 10^5/0.2 ml/well) were cultured with PHA (8 μg/ml) for 48 h. After culture, the cells were pulsed with [^{3}H]TdR for 4 h.

DISCUSSION

The present results demonstrate that CTS mouse spleen cells are markedly reduced in a variety of in vitro T cell responses. The proliferative responses to T cell mitogens and alloantigens are very meager, if any exists, and the production of IL-2 and IL-2 receptors are also greatly diminished. Because T cell-enriched spleen cells responded normally to PHA, the reduction in T cell activity

seems primarily to depend on the decrease in the number of T cells and not on the dysfunction of individual T cells. This speculation was supported by the flow cytometry of spleen cells, which revealed that T cells, particularly Lyt 2^+ T cells, are greatly reduced in number. However, restoration of the Con A response by T cell enrichment was only partial, in comparison with the full restoration of PHA response. Therefore, dysfunction of T cells could not be neglected as far as Con A response is concerned. Although decrease in Lyt 2^+ T cells was also observed with the NON mouse splenocytes, no abnormality was seen with regard to the mitogen responses. This seems to be due to the existence of the normal level of L3T4$^+$ T cells.

Our present examination revealed that the NOD mouse was not T-lymphocytopenic. This is in conflict with an earlier report by Kataoka et al. (69). However, T-lymphocytopenia does not seem to be the case with the ordinary NOD mouse, because their own re-examination study (70) and a fluorescent microscopic study by others (71) did not confirm it. This disagreement with regard to the NOD mouse may be attributed to the difference in the mouse colonies used: T cell deficiency was proposed with a peculiar colony in which the cumulative incidence of spontaneous diabetes around 30 weeks of age was less than 20% even in the females. In contrast, T cell deficiency is definite in the CTS mouse. Some researchers have demonstrated that viral and/or bacterial infections cause impairment of T cell activity (72). In our case, this possibility could be ruled out, because the CTS mice had been kept under a specific pathogen-free condition until the spleen cell harvest. According to our preliminary study, a profound decrease in T cells was also observed in peripheral blood and in the lymph nodes, but much more mature T cells, i.e., L3T4-single positive cells, were located in the thymus medulla of the CTS mice than of the reference strains. Taken together, migration of mature T cells from thymus to peripheral lymphoid tissue appears to be impaired in the CTS mouse.

Strain difference was also obvious in NK activity. The NON mouse displayed high NK activity comparable with that of the C3H/He mouse which is known as an NK-high responder. The CTS mouse had moderate degree of NK activity but the NOD mouse did not show significant activity. Because flow cytometric analysis demonstrated that the percentages of asialoGM_1^+ cells did not vary

very much among these three strains, the difference could be due to that in the function. Although the in vivo role of NK cells is not so definite, β-cell killing is proposed in the BB rat (73, 74) and NOD mouse (71). However, the present results suggest that this is not the case at least with the NOD mouse.

VI. Immune Deficiency of CTS Mouse: Impaired in vivo T Cell-Mediated Immune Response

INTRODUCTION

Non-obese diabetic (NOD) mice have been established at Shionogi Aburahi Laboratories from an outbred colony of Jcl:ICR mice as an animal model for human type I diabetes mellitus. Several strains such as CTS (cataract Shionogi) and NON (non-obese non-diabetic) mice have been isolated as sister strains of NOD mice. Detailed description of their mutual relationship is given elsewhere (59). Our genetical analysis of the pathogenesis of diabetes in NOD mice revealed that two recessive genes are responsible for the development of insulitis (60), one of which is associated with the MHC (particularly class II) gene complex (62, 63). Recently class II genes of the CTS mice have been shown to be identical with those of the NOD mouse (65, 75), and therefore, it is easily understood that the CTS mice carry the MHC-associated gene of NOD mice (67). On the other hand, genetical study using F_1, F_2 and backcross offspring between the NOD and NON strains suggests that these two strains share allele of the MHC-non-associated gene (67). In view of these findings, it would be of some value to study the biological, especially immunological, characteristics of the CTS and NON strains, since such a study might provide an insight into the pathogenic mechanism of diabetes in the NOD mouse. My study along this line elucidated, as described in Chapter V, that the CTS mice are T-lymphocytopenic and display marked impairment of in vitro T cell-mediated immune responses such as proliferation of splenic lymphocytes upon stimulation with Con A, PHA or alloantigens, and production of IL-2 and IL-2 receptors after Con A-stimulation. To obtain further evidence of T cell deficiency in the CTS mouse, the present study deals with the in vivo immune responses to thymus-dependent and -independent antigens.

RESULTS

Table 6–1

Primary antibody response to a high dose (10^8) of SRBC

Strain (Sex)	Antibody positivity[a] (mean titer±SD)[b]	
	Total (IgM)	2ME–resist. (IgG)
CTS (F)	6/6 ($2^{5.3 \pm 0.7}$)*	1/6 (2^0)
CTS (M)	7/7 ($2^{6.6 \pm 0.5}$)*	4/7 ($2^{0 \pm 0}$)*
NOD (F)	3/3 ($2^{9.2 \pm 0.5}$)	3/3 ($2^{6.3 \pm 0.3}$)
NOD (M)	5/5 ($2^{8.4 \pm 0.8}$)	5/5 ($2^{5.8 \pm 1.0}$)
NON (F)	7/7 ($2^{9.7 \pm 1.5}$)	7/7 ($2^{6.7 \pm 0.5}$)
C3H/He (F)	3/3 ($2^{10.7 \pm 0.9}$)	3/3 ($2^{8.7 \pm 0.9}$)
DS (F)	3/3 ($2^{10.7 \pm 0.5}$)	3/3 ($2^{7.3 \pm 0.5}$)
BDF_1 (F)	8/8 ($2^{8.1 \pm 0.4}$)	8/8 ($2^{7.6 \pm 0.4}$)

a) Number of the antibody–positive sera/number of sera tested.

b) Geometric mean titer of the antibody–positive sera

*) Significantly lower ($p < 0.001$) compared with the antibody titer of each reference strain

Antibody responses to SRBC. Table 6–1 shows the serum antibody titers one week after an intravenous injection of 10^8 SRBC. Production of IgM antibody, expressed in terms of the total antibody titers, was observed in CTS mice, as well as in the reference strains. However, the antibody titers were significantly lower ($p < 0.001$) in CTS mice than in the others. Production of IgG antibody (2–ME–resistant titer) was marginal, if any, in CTS mice, although it was definite in all the other

Table 6–2

Primary and secondary antibody responses to a low dose (10^5) of SRBC

Immune response	Strain (Sex)	Antibody positivity[a] (mean titer±SD)[b]	
		Total (IgM)	2ME–resist. (IgG)
Primary	CTS (F)	0/6	0/6
	CTS (M)	0/5	0/5
	NOD (F)	4/4 ($2^{6.5 \pm 0.5}$)	0/4
	NON (F)	5/5 ($2^{7.8 \pm 1.2}$)	3/5 ($2^{8.7 \pm 0.9}$)
Secondary	CTS (F)	6/6 ($2^{6.7 \pm 1.1}$)*	0/6
	CTS (M)	6/6 ($2^{6.3 \pm 1.0}$)*	0/6
	NOD (F)	5/5 ($2^{10.2 \pm 0.7}$)	5/5 ($2^{6.4 \pm 0.5}$)
	NON (F)	10/10 ($2^{10.2 \pm 0.7}$)	10/10 ($2^{7.4 \pm 0.9}$)

a) Number of the antibody–positive sera/number of sera tested.

b) Geometric mean titer of the antibody–positive sera

*; Significant decrease ($p < 0.001$) compared with the antibody titer of each reference strain

reference strains. Thus, the difference was more striking with the IgG response than with the IgM response. Similar results were obtained in the case of the primary and secondary responses to a low dose (10^5) SRBC (Table 6–2). Marked difference was observed between CTS and its two sister strains, i.e., NOD and NON, the difference being particularly obvious with regard to the IgM (total) titer at the primary response and the IgG (2–ME–resistant) titer at the secondary response.

Antibody response to BSA. As demonstrated in Table 6–3, both IgE and IgG_1 anti–BSA antibodies were produced at high incidence in all the reference strains one to three weeks after the immunization with BSA plus Bp., although somewhat in DS mice. In contrast, production of the

Table 6–3

Antibody response to a single injection of BSA plus Bp

Immunizing period	Strain	Sex	Antibody positivity[a] (mean titer±SD)[b]	
			IgE	IgG_1
1 week	CTS	F	0/5	0/5
		M	0/15	1/15 ($2^{0.0}$)
	NOD	F	14/15 ($2^{3.6 \pm 0.5}$)	7/7 ($2^{4.7 \pm 1.0}$)
		M	5/5 ($2^{4.8 \pm 1.0}$)	5/5 ($2^{5.0 \pm 0.0}$)
	NON	F	5/5 ($2^{1.8 \pm 1.0}$)	5/5 ($2^{2.8 \pm 0.4}$)
	C57BL/6	F	7/7 ($2^{4.1 \pm 0.7}$)	1/7 ($2^{2.0}$)
	BALB/c	F	5/5 ($2^{3.8 \pm 0.4}$)	5/5 ($2^{4.6 \pm 0.5}$)
	C3H/He	F	10/10 ($2^{3.4 \pm 0.5}$)	9/10 ($2^{2.8 \pm 0.4}$)
	DS	F	3/10 ($2^{0.0 \pm 0.0}$)	1/10 ($2^{0.0}$)
	BDF_1	F	5/5 ($2^{6.6 \pm 0.5}$)	5/5 ($2^{6.8 \pm 0.0}$)
2 weeks	CTS	F	1/14 ($2^{0.0}$)	13/14 ($2^{1.8 \pm 1.4}$)*
		M	0/7	7/7 ($2^{3.3 \pm 1.0}$)*
	NOD	F	9/9 ($2^{4.2 \pm 0.6}$)	9/9 ($2^{8.2 \pm 0.5}$)
		M	4/4 ($2^{3.2 \pm 0.4}$)	6/6 ($2^{9.0 \pm 0.0}$)
	NON	F	9/9 ($2^{2.0 \pm 0.7}$)	9/9 ($2^{5.4 \pm 1.5}$)
		M	3/3 ($2^{1.7 \pm 0.5}$)	3/3 ($2^{6.7 \pm 0.9}$)
	C57BL/6	F	6/6 ($2^{4.3 \pm 0.5}$)	6/6 ($2^{5.5 \pm 1.3}$)
	BALB/c	F	5/5 ($2^{4.0 \pm 0.7}$)	5/5 ($2^{4.8 \pm 0.4}$)
	C3H/He	F	4/5 ($2^{3.0 \pm 0.0}$)	5/5 ($2^{6.4 \pm 0.8}$)
	DS	F	8/8 ($2^{0.5 \pm 0.5}$)	8/8 ($2^{3.9 \pm 0.3}$)
	BDF_1	F	6/6 ($2^{4.6 \pm 1.4}$)	6/6 ($2^{6.4 \pm 1.4}$)
3 weeks	CTS	F	0/5	5/5 ($2^{4.8 \pm 0.8}$)*
	BALB/c	F	6/6 ($2^{4.2 \pm 0.9}$)	6/6 ($2^{8.8 \pm 0.4}$)
	DS	F	4/5 ($2^{1.7 \pm 1.4}$)	5/5 ($2^{5.8 \pm 0.9}$)
	BDF_1	F	5/5 ($2^{9.4 \pm 0.5}$)	5/5 ($2^{7.6 \pm 0.5}$)

a) Number of the antibody-positive sera/number of sera tested.
b) Geometric mean antibody titers of the antibody-positive sera
*; Significant difference ($p < 0.001$) compared with the antibody titer of the reference strains except for DS mouse

antibodies was very meager in CTS mice: only a low titer of IgG_1 antibody was detected at two to

three weeks of immunization and no IgE antibody was found throughout the test period up to three weeks, except for one mouse displayed minimum activity.

Table 6–4

Production of active systemic anaphylaxis to BSA

Strain	Sex	Incidence of lethal shock at	
		1 week	2 weeks
CTS	F	14/15	15/15
	M	7/10	12/12
NOD	M	12/14	12/15
C57BL/6	F	0/12	6/6
BALB/c	F	9/10	12/20
C3H/He	F	11/11	10/10
DS	F	1/10	11/11
BDF_1	F	11/12	9/9

Production of active systemic anaphylaxis. In spite of the poor production of circulatory anti–BSA antibodies, lethal active anaphylactic shock was produced in almost all the CTS mice, as well as in the other strains (Table 6–4). It should be noticed that lethal shock was provoked in most CTS mice one week after the immunizing injection, even though neither IgE or IgG_1 antibody was detected in their sera. This stands in contrast with the finding that no shock symptoms were observed at one week of immunization in C57BL/6J mice carrying serum IgE antibody.

Shultz–Dale reaction. To support the result that lethal shock was easily provoked in CTS mice irrespective of the impaired production of circulating homocytotropic antibodies, Shultz–Dale reaction was tested using uterus muscle obtained from the CTS mice immunized with BSA plus Bp for two

Table 6–5

Correlation between active Schultz–Dale Reaction and serum antibody titers

Animal No.	Schultz–Dale reaction	Serum antibody titer	
		IgE	IgG_1
#1	Positive	ND*	2^2
#2	Positive	ND	2^2
#3	Positive	ND	2^0
#4	Negative	ND	ND
#5	Positive	2^0	ND

*; ND: Not detected

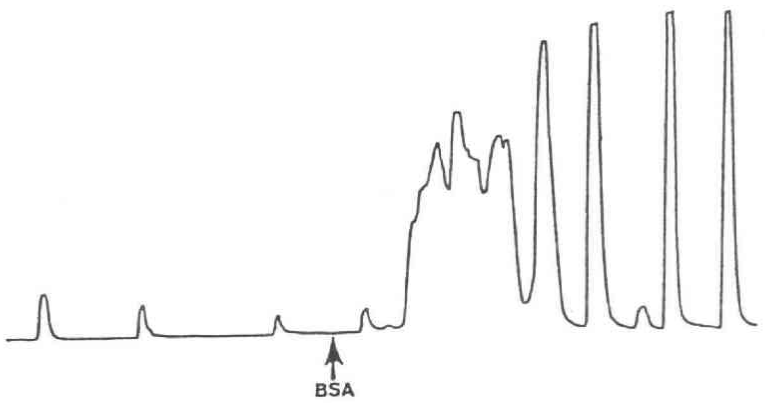

Fig. 6–1. Shultz–Dale reaction of the uterus muscle prepared from CTS mouse #3 sensitized actively to BSA for 2 weeks.

weeks. As shown in Table 6–5, the uterus from four out of five mice contracted upon treatment with the antigen. Fig. 6–1 illustrates the contraction of the uterus from the animal #3, which displayed no IgE antibody and only minimum IgG_1 antibody activity.

Table 6–6

Delayed type of hypersensitivity to SRBC

Strain (Sex)	Immunogen	n	Footpad swelling (x 10^{-2} mm)	
			28–hour	48–hour
CTS (F)	SRBC	6	49 ± 17.2[a]	21 ± 13.0
	PBS	6	23 ± 10.1	5 ± 4.6
			(26)[b]	(16)
CTS (M)	SRBC	4	56 ± 30.1	16 ± 15.1
	PBS	4	24 ± 5.0	6 ± 5.7
			(32)	(10)
NOD (F)[c]	SRBC	6	129 ± 17.3	81 ± 16.1
	PBS	8	15 ± 4.9	24 ± 7.3
			(114)	(57)
NON (F)	SRBC	6	87 ± 7.8	29 ± 7.0
	PBS	6	32 ± 8.0	0 ± 0
			(55)	(29)
C57BL/6 (F)	SRBC	5	154 ± 26.4	74 ± 19.8
	PBS	5	20 ± 7.9	9 ± 5.3
			(134)	(65)
BDF_1 (F)	SRBC	8	131 ± 17.8	72 ± 11.6
	PBS	8	19 ± 5.9	12 ± 5.6
			(112)	(60)

a) Mean ± SD
b) Difference between experiment and control, i.e., the increment due to delayed type of hypersensitivity.
c) 16 weeks of age

Delayed type of hypersensitivity to SRBC. As demonstrated in Table 6–6, 28–hour swelling of the footpads was only slight in CTS mice, while definite in the reference strains. Forty eight hours after the antigen challenge, swelling diminished in all the strains tested, but the difference between CTS and reference strains was still observed.

Table 6–7

Skin graft rejection

Host	Rejection time in days (mean ± SD) from donors				
	CTS	NOD	NON	C3H/He	C57BL/6
CTS	>31	>31	17 ± 1.4	13 ± 1.9	21 ± 9.3
	(17)	(15)	(6)	(10)	(10)
NOD	18 ± 3.7	>31	21 ± 1.6	14 ± 3.7	16 ± 5.0
	(14)	(55)	(20)	(11)	(9)
NON	13 ± 2.6	19 ± 6.2	>36	15 ± 4.8	12 ± 2.7
	(5)	(20)	(43)	(13)	(8)
C3H/He	10 ± 0	15 ± 6.2	13 ± 3.4	>31	12 ± 2.7
	(10)	(4)	(15)	(8)	(10)
C57BL/6	12 ± 4.3	15 ± 4.7	14 ± 2.3	20 ± 7.6	>31
	(10)	(9)	(5)	(3)	(10)

Number of the mice tested including both sexes given in parenthesis.

Skin graft rejection. Reciprocal skin transplantation was performed using both sexes of CTS and other strains at the age of 5 weeks. As shown in Table 6–7, skin grafts from different strains were not accepted in general. The time for the rejection did not vary very much in all the order–recipient combinations. Only one exceptional case was the failure of CTS mice to reject the grafts from NOD mice within 31 days.

Antibody response to LPS. The above results indicated that in vivo immune responses to T–dependent antigens are markedly impaired in CTS mice. In contrast, nearly the same degree of antibody response was observed against lipopolysaccharide (LPS) in CTS and other strains (Table 6–

Table 6-8

Antibody response to LPS

Strain (Sex)	Immunogen	Bleeding time	Antibody positivity[a] (Mean titer±SD)[b]	
			Total (IgM)	2ME-resistant (IgG)
CTS (F)	LPS	Day 4	4/4 ($2^{6.1 \pm 0.9}$)	4/4 ($2^{3.0 \pm 0.7}$)
		Day 7	4/4 ($2^{5.8 \pm 0.5}$)	4/4 ($2^{5.8 \pm 0.8}$)
		Day 14	5/5 ($2^{6.6 \pm 0.5}$)	5/5 ($2^{5.2 \pm 0.5}$)
NOD (F)	LPS	Day 4	6/6 ($2^{6.6 \pm 1.2}$)	0/6
DS (F)	LPS	Day 4	5/5 ($2^{6.2 \pm 0.7}$)	4/5 ($2^{1.6 \pm 1.0}$)
		Day 7	5/5 ($2^{6.0 \pm 0.0}$)	5/5 ($2^{6.2 \pm 0.4}$)
BALB/c nude (F)	LPS	Day 4	8/8 ($2^{7.6 \pm 0.6}$)	6/8 ($2^{1.6 \pm 1.0}$)
		Day 7	8/8 ($2^{9.2 \pm 0.7}$)	8/8 ($2^{4.0 \pm 1.0}$)
		Day 14	8/8 ($2^{7.8 \pm 0.4}$)	8/8 ($2^{4.0\ 0.9}$)
CTS (F)	None		2^{2}[c]	ND[d]
DS (F)	None		2^{2}[c]	ND[d]

a) Number of the antibody-positive sera/Number of sera tested

b) Geometric mean titer of the antibody-positive sera

c) Titer of the pooled serum of five mice

d) ND: Not detected

8). Definite hemagglutinin titers were obtained with the antisera of any strain against LPS-coated SRBC but not against uncoated SRBC.

DISCUSSION

In chapter V, I demonstrated that CTS mice are T-lymphocytopenic and therefore deficient in the in vitro responsiveness to T cell mitogens, such as Con A and PHA, but not to a B cell mitogen,

LPS. The present in vivo results are consistent with these in vitro findings. The antibody responses to T-dependent particulate and soluble antigens, i.e., SRBC and BSA, respectively, were markedly lower, but the response to LPS was essentially normal. Delayed type of hypersensitivity was also impaired, and rejection of the skin grafts from NOD mice was significantly delayed.

With regard to the production of anti-SRBC antibodies, reduction was more striking with IgG response than with the IgM response. Using thymectomized and irradiated mice, it has been demonstrated that the cells producing IgG antibody to SRBC are more dependent on the presence of thymus than those that produce IgM antibody (76). This is supported by our unpublished data that some amount of anti-SRBC IgM, but not IgG, antibody is produced by BALB/c athymic nude mice. In view of these findings, the more profound reduction of anti-SRBC IgG response than of IgM response in the T cell-deficient CTS mice is not unexpected. Similar deficient antibody response was observed in CTS mice against BSA. This was the case with both the IgG_1 and IgE responses. Throughout the test period, no IgE antibody was detected by rat PCA, except for in one mouse that showed minimal IgE antibody activity. An extremely low IgE response seems to be primarily due to the deficiency of the IgE class-specific helper T cells leading to diminished production of IL 4, although the possibility that IgE-producing B cells are also decreased cannot be ruled out.

An unexpected finding concerning the anti-BSA response is that lethal anaphylactic shock was easily provoked in CTS mice irrespective of the poor production of homocytotropic antibodies. The discrepancy could be explained assuming that CTS mice are highly susceptible to the effector phase of active anaphylactic shock. In this strain, release of small amount of chemical mediators from mast cells sensitized with minute amount of antibodies produced would be sufficient to kill them. On the other hand, C57BL/6J mice did not undergo lethal shock one week after the immunization, even though they had circulatory IgE antibody. Thus, it is difficult to predict the occurrence of anaphylactic shock in the mice on the basis of serum IgE and IgG_1 antibody levels.

In contrast with the marked reduction of antibody response to T-dependent antigens, no difference was observed between CTS and other strains in the antibody production to LPS. In the

present study, 2-ME-resistant antibody titers were observed one to two weeks after the immunization, though only low, if any, titers were seen four days after the immunization. So far as the available information is concerned (77, 78), the antibody elicited by a single dose of LPS is exclusively of the IgM class. However, because there is no evidence of the occurrence of 2-ME-resistant IgM, the 2-ME-resistant anti-LPS antibody observed in the present study seems to be of the IgG class. If this is the case, class switch from IgM to IgG could take place in the primary response against LPS, as well as against T-dependent antigens. The present results demonstrated that nearly the same degree of the anti-LPS IgG response could occur in the T cell-deficient CTS mice as in the reference strains, which would conflict with the above described high T cell-dependency of the anti-SRBC IgG response. The reason for the discrepancy awaits to be explained by further studies.

Cell-mediated immunity was also decreased in CTS mice. It is easy to understand that DTH to SRBC was very weak in this mouse strain. Since the cell-mediated immunity to a low dose of SRBC is only transient (79, 80), T cell-deficiency could exert a great influence on it. On the other hand, rejection of allogeneic skin pieces was not affected in CTS mice in general. This appears to be inconsistent with the results described in Chapter V that in vitro proliferative responses and generation of killer T cells to alloantigens were remarkably diminished with the spleen cells of this strain. But, the discrepancy could be explained by taking into account the continued antigenic stimulation in the in vivo skin transplantation in contrast to a relatively short term co-culture of the responding cells with the stimulator cells in mixed lymphocyte reaction. However, CTS mice did not reject the grafts from NOD mice. According to the some authors (65, 75), these two strains have the very similar MHC; both have the same class II and semiidentical class I K-end, though different in class I D-end. Such a similarity of MHC in addition to the T cell-deficiency could be the cause for the failure or delay of the rejection. In NOD mice, which rejected CTS skin, even a small difference in class I MHC would be enough to raise the cell-mediated immunity, because they have normal level of T cell function.

At present, it is unclear whether T cell-deficiency and diminished immune responses correlate

with the development of cataract eyes in CTS mice. It is also unclear whether the present findings with CTS mice provide any insight into the pathogenic mechanism of NOD mice. But, marked T cell-deficiency in CTS mice suggests that this strain is a good tool for the study of T cell maturation and distribution. Our preliminary study revealed that much more mature T cells, particularly $L3T4^+$ $Lyt\ 2^-$ cells, are retained in the thymus medulla in CTS mice than in the other strains. This suggests that homing of mature T cells in peripheral lymphoid tissues might be impaired in this mouse strain.

VII. Conclusions

1) Culture of hydrocortisone (HC)-resistant C57BL/6 mouse thymocytes with recombinant human interleukin 2 (IL-2) allowed the proliferation of the thymocytes and resulted in the generation of lymphokine-activated killer (LAK) cells cytotoxic to a variety of tumor cells. The cytotoxic activity of the LAK cells was greatly reduced by treatment with anti-Thy 1.2 or anti-Lyt 2.2 monoclonal antibody and complement but not with anti-asialoGM_1 antibody plus complement. Fractionation of IL-2-stimulated thymocytes by means of Percoll density gradient centrifugation revealed that both cytotoxic activity and binding capacity to target cells were greater in the cells with lower density and larger size than in the cells with higher density and smaller size. These IL-2-activated thymocytes expressed higher levels of both Thy 1, Lyt 2 and lymphokine-activated cell-associated (LAA) antigens than unstimulated thymocytes, as indicated by a flow cytometric analysis. The frequency of LAK precursor cells was found to be 7.5 times greater in the HC-resistant thymocyte population than in total thymocytes, as determined by means of a limiting dilution method. The LAK precursor cells in HC-resistant thymocytes appeared to be Lyt 1^- (or dull Lyt 1^+), L3T4$^-$, Lyt 2^- T cells, because elimination of bright Lyt 1^+, Lyt 2^+ or L3T4$^+$ T cells from HC-resistant thymocytes had no effect on the generation of LAK cells. These results indicate that LAK cells from mouse thymocytes are Lyt 2^+ T cells which are inducible from HC-resistant Lyt 2^- thymocytes.

2) It has previously been shown that killer-blocking monoclonal antibody (KBA mAb) recognizes lymphokine-activated cell-associated antigen (LAA) involved in broad-reactive killer (BRK) cell-mediated cytotoxicity. I now reported that LAA is expressed on all lymphoid cells, though the amount of LAA on unstimulated lymphocytes is low. In contrast, lymphocytes activated in vitro with either concanavalin A, alloantigens, lipopolysaccharide, or recombinant interleukin 2 express high levels of LAA. In addition, in vivo activated lymphocytes, such as OK-432-activated lymphocytes and tumor-infiltrating lymphocytes express higher levels of LAA than unstimulated lymphocytes. I also

demonstrated that the expression of LAA is restricted in T-cell lymphomas and a Mϕ cell line, while myelomas, fibrosarcomas, and carcinomas do not express LAA. Cell cycle analysis using propidium iodide and KBA mAb showed that LAA expression was closely correlated with the transition of cells from G_{1a} to G_{1b} phase.

3) The addition of monoclonal killer blocking antibodies (KBA mAb) to cultured T cells resulted in significant inhibition of T-cell responses to concanavalin (Con A), class I antigen and class II antigen, whereas T-cell responses to phytohemagglutinin are insensitive to KBA mAb. The inhibitory effect of KBA mAb is observed only when KBA mAb is added to the culture at an early time. This indicates that the lymphokine-activated cell-associated antigen (LAA) defined by KBA mAb plays an important role in T-cell activation at the early stages. Con A-induced interleukin 2 (IL-2) receptor acquisition and IL-2 production, both of which are required for the early steps of T-cell activation, were greatly inhibited by KBA mAb. However, KBA mAb did not inhibit the action of IL-2, which is required for later stages of T-cell activation.

4) The cataract Shionogi (CTS) mouse characterized by cataracts and microphthalmia is a sister strain of the NOD mouse. I have made the immunological characterization of the CTS mouse by means of in vitro assays. Splenocytes of the CTS mouse were very low in the responsiveness to T cell mitogens such as Con A and PHA but not to a B cell mitogen, LPS. The production of IL-2 and expression of IL-2-receptor of spleen cells after in vitro stimulation with Con A decreased in the CTS mouse, when compared with those in the NOD and the other reference strains. In mixed lymphocyte culture, CTS splenocytes did not proliferate and did not generate cytotoxic T lymphocytes when cocultured with splenocytes of the C3H/He mouse. The NK activity against YAC-1 target cells was lower in the CTS mouse than in the C3H/He mouse, an NK high responder, but higher than in the NOD mouse, a low responder. These results suggest that the CTS mouse is deficient in T cells. Subset analysis of splenic lymphocytes of the CTS mouse using flow cytometry revealed that the percentage of T cells

in the CTS mouse was significantly lower than those in the reference strains, which was consistent with the reduced responsiveness to T cell mitogens in the CTS mouse. The deficiency in the Lyt 2^+ T cell subset was particularly striking. However, the response to PHA of the splenocytes of the CTS mouse was normalized when T cells were enriched by nylon wool–passing and cell–sorting. Therefore, it seems that decreased T cell activity is due to a decrease in T cell number and not to dysfunction of individual T cells.

5) To confirm in vitro characteristics, in vivo immune responses of CTS mice to T–dependent and T–independent antigens were compared with those of some reference strains including NOD mice. Antibody responses of CTS mice after one injection of a high dose (10^8) or one or two injections of a low dose (10^5) of sheep red blood cells (SRBC) were markedly lower than those of the reference strains. The decrease was particularly striking in the IgM antibody production at primary response to both high and low doses, and the IgG antibody production at the secondary response to low dose. Similar lower antibody production was observed in CTS mice against bovine serum albumin (BSA). Little production of IgE antibody was observed from 1 through 3 weeks after an injection of BSA plus Bordetella pertussis. IgG_1 response was observed at high incidence but lower in titer than those in the reference strains. Unexpectedly, in spite of the poor antibody production to BSA, potent systemic sensitization for anaphylactic shock was easily established; incidence of lethal shock being comparable with those in the reference strains. This suggests that CTS mice are highly susceptible to the effector phase of active anaphylactic shock. Cell–mediated immunity was also impaired. Delayed type of hypersensitivity to SRBC was low, and the rejection of the skin graft from NOD mouse did not occur. In contrast to the reduced T cell–mediated responses, no difference was found between CTS and reference strains with regard to the antibody production to LPS, a T–independent antigen. These in vivo findings are consistent with the in vitro study.

VIII. Materials and Methods

Animals. Male C57BL/6 mice were obtained from Funabashi Farm, Chiba, and used at ages of 5–8 weeks (Chapter II–IV). CTS/Shi, NOD/Shi, NON/Shi, DS/Shi, AKR, CBA/N, C57BL/6, BALB/c and C3H/He female mice were used at ages of 8 to 15 weeks (Chapter V). Both sexes of CTS, NOD and NON strains, and females of C3H/He, C57BL/6J, BALB/c, DS/Shi, (B6 x DBA/2)F_1, and BALB/c athymic nude mice were used (Chapter VI). All the mice in Chapter V–VI were bred at Shionogi Aburahi Laboratories under specific pathogen–free condition, and transferred to a clean but conventional condition at the time of starting the experiments. Unless otherwise described, the animals were used for immunization at 7 to 9 weeks of age in the case of Chapter VI. Female DS/Shi mice and WS rats (bred at Shionogi Aburahi Laboratories as an inbred strain, 7 to 8 weeks of age) were employed as the recipients for passive cutaneous anaphylaxis (PCA).

Tumor cells. BMC2 ($H-2^b$), BMC6A ($H-2^b$), and Meth A–ad (adherent subclone of Meth A, $H-2^d$) fibrosarcomas, MBL–2 ($H-2^b$), EL 4 ($H-2^b$), FBL–3 ($H-2^b$), YAC–1 ($H-2^a$), RADA1 ($H-2^a$) and BW5147 ($H-2^k$) lymphomas, P3X63Ag.853 ($H-2^d$), X5563 ($H-2^k$) and SP2/0–Ag14 ($H-2^d$) myelomas, $P388D_1$ ($H-2^d$) macrophage cell line, MM48 ($H-2^k$) mammary tumor and B16 ($H-2^b$) melanoma were used in these studies. These tumor cells were maintained by in vitro culture with RPMI1640 medium containing penicillin G at 100 units/ml, kanamycin sulfate at 60 μg/ml, 1 μM sodium pyruvate, HEPES at 2.3 mg/ml, $NaHCO_3$ at 1 mg/ml, and 10% fetal calf serum (FCS) (Filtron Pty, Altona, Australia).

Interleukin 2. rh–IL–2 having specific activity of 1.4×10^7 JU (Jurkat unit)/mg was kindly donated by Shionogi & Co., Ltd., Osaka, and used after appropriate dilution (81, 82).

Monoclonal antibodies (mAbs). KBA Hybridoma cells were maintained by in vivo passage in nude mice. The ascitic fluid containing KBA mAb was harvested and purified by 50% ammonium sulfate precipitation twice. This preparation of KBA mAb (IgG_{2a}) gave a single band of protein in SDS–PAGE.

Fluorescein isothiocyanate (FITC)-conjugated monoclonal anti-Thy 1.2 (30-H12), anti-Lyt 1 (53.7.3), anti-Lyt 2 (53.6.7) and anti-mouse IgM (331.12) antibodies were purchased from Becton Dickinson Monoclonal Center, Inc., CA. FITC-conjugated monoclonal anti-Thy 1.1 (OX-7) antibody was purchased from Serotec Ltd., Blackthorn Bicester, England. Monoclonal anti-Mac-1 antibody was purchased from Hybritech Inc., CA. FITC-conjugated monoclonal anti-rat kappa (MARK-1) antibody and FITC-conjugated anti-rabbit IgG antibody were purchased from Cosmo Bio Inc., Tokyo, Japan and DAKOPATTS A/S, Glostrup, Denmark, respectively. FITC-conjugated monoclonal anti-L3T4 (GK-1.5) and anti-mouse interleukin 2-receptor (3C7) antibodies and anti-asialoGM_1 antibody were kindly donated by Drs. K. Okumura and H. Yagita of Juntendo University, Tokyo, Japan.

Antigens. Sheep red blood cells (SRBC, Nacalai Tesque Inc., Kyoto, Japan) and bovine serum albumin (BSA, Nakarai Chemicals, Kyoto, Japan) were used as thymus-dependent antigens, and lipopolysaccharide of Escherichia coli (LPS, Difco, Detroit, Mich., USA) as a thymus-independent antigen. These antigens were suspended or dissolved in phosphate-buffered saline solution (PBS, Ph 7.0).

Preparation of thymocytes. Thymocytes were obtained from C57BL/6 mice before or 24 h after an intraperitoneal injection of a suspension of hydrocortisone acetate (Wako Pure Chemical Industries, Osaka) in physiological saline.

Preparation of activated lymphocytes. In vitro activated lymphocytes were obtained from C57BL/6 mouse spleen cells by incubating with Con A (2.5 μg/ml) or LPS (40 μg/ml) for 48 h at 37°C. In vivo activated lymphocytes were prepared from peritoneal exudate cells (PEC) of C57BL/6 mice which were treated with 1 KE/mouse of Streptococcus preparation of OK-432 (generously donated by Chugai Pharmaceutical Co., Ltd., Tokyo) 3 days before the experiment. To remove macrophages, PEC were treated with plastic dish and nylon wool column (83) before the experiment.

Preparation of tumor-infiltrating lymphocytes (TIL). BMC2 tumor cells (10^7) were injected sc into C57BL/6 mice. This resulted in the development of tumor nodules 15 days after the injection. Tumor tissues of BMC2 were cut into 2- to 3-mm^3 fragments in phosphate-buffered saline (PBS),

washed twice with PBS, and digested with 500 U/ml of Dispase (Godo Shusei, Co., Ltd., Tokyo) at 37°C for 20 min. Tumor-infiltrating lymphocytes were prepared from these tumor cell suspensions using glass wool columns as described previously (27).

Preparation of spleen cells. Spleens from CTS and other mice were removed, teased apart, and pressed through mesh, using Hanks' balanced salt solution (HBSS, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) to produce a single-cell suspension. Lymphocytes were isolated by density gradient centrifugation (at 1,200 x *g* for 20 min) using a lymphocyte separation medium, M-SMF (JIMRO, density: 1.090, Nihon Kotai Kenkyu-sho, Takasaki, Japan), and then washed HBSS three times. Viable cells were counted by the trypan blue exclusion test (viability: more than 95%) and adjusted to appropriate cell concentrations with RPMI1640 medium (Flow Laboratories Inc.) containing 10% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories Inc., UT).

Assay for proliferative response of thymocytes to IL-2. The assay was performed according to the method described in a previous paper (28). Briefly, aliquots (0.2 ml) of cell suspensions (2.5 x 10^6 cells /ml) were cultured with rh-IL-2 (2000 JU/ml) in a Costar No. 3599 plastic plate for various periods at 37°C. The cells were pulsed with ^{3}H-thymidine (^{3}H-TdR) (Amersham Japan, Tokyo) for 4 h at 37°C, collected with the aid of a Skatron cell harvester, and counted for radioactivity by a standard liquid scintillation technique.

Induction of killer cells and assay for cytotoxicity. LAK, NK cells and CTL were prepared by the method described previously (10). In the case of thymocyte-LAK, thymocytes (5 x 10^6) from HC-treated or untreated C57BL/6 mice were cultured in the presence of rh-IL 2 (2000 JU/ml) in a Costar 12-well plastic plate for 4-5 days at 37°C. The cells were collected and assayed for cytotoxic activity by the ^{3}H-uridine method as reported previously (84, 85). Briefly, an aliquot (0.1 ml) of an effector cell suspension was mixed with a suspension of ^{3}H-uridine-labeled target cells (4 x 10^3 cells in 0.1 ml) in a Costar No. 3799 U-bottomed plastic plate, and the cell mixture was cultured for 18 h at 37°C. The cells were collected by the use of a Skatron cell harvester, and their radioactivity was measured by a standard liquid scintillation technique.

The percentage of target cell lysis was calculated from the following equation: % cytotoxicity = 100 x (1 – cpm of target cells cultured with effector cells / cpm of target cells cultured alone). For standardization of cytotoxicity, the number of effector cells required for 25% lysis of 4 x 10^3 target cells was defined as 1 lytic unit (LU) (Chapter II–IV).

In the case of Chapter V, target cells for alloimmune CTL were the Con A–induced splenic blast cells. The YAC–1 cell line was used as a target for NK cytolysis. Approximately 5 x10^6 target cells were incubated with 0.1 mCi of $Na_2{}^{51}CrO_4$ for 1.5 h at 37°C, washed three times, and adjusted to 1 x 10^5/ml. A 0.1 ml portion containing 1 x 10^4 cells was poured into each well of 96–well round–bottomed plates (Linbro, Flow Laboratories Co., Virginia) with 0.1 ml of effector cell suspensions containing different numbers of effector cells; each dilution of the effector cell suspension was plated in triplicate. After incubation at 37°C for 4 h, a 100–µl aliquot of culture supernatant was removed from each well for a radioactivity count. Spontaneous release of ^{51}Cr was measured in the absence of effector cells (in RPMI1640 medium containing 10% FCS instead of the effector cell suspension), and total release was measured using the detergent–lysed target cells. The percentage of cytotoxicity was calculated using the following formula:

$$\frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Total release} - \text{Spontaneous release}} \times 100$$

For standardization of cytotoxicity, the number of effector cells required for 25% lysis of 1 x 10^4 target cells was defined as one lytic unit (LU).

Flow cytometry for assay of cell surface phenotypes. Fluorescein isothiocyanate (FITC)–conjugated monoclonal antibodies against Thy 1.2, Lyt 1 or Lyt 2, phycoerythrin (PE)–conjugated monoclonal antibody against L3T4 antigen (Becton–Dickinson), and monoclonal antibody (KBA) (10) against LAA antigen were used for assays. A cell pellet of 10^6 cells was incubated with 10 µl of FITC–conjugated antibody or biotinylated monoclonal antibody (KBA) for 45 min under ice–cooling. For two–color staining, PE–conjugated antibody was added together with FITC–conjugated antibody. After incubation, the cells were washed 3 times with phosphate–buffered saline, PH 7.2 (PBS), treated

(in the case of KBA) or untreated with FITC–conjugated avidin (Becton–Dickinson) for 45 min at 4°C, and then analyzed for the staining pattern by flow cytometry in a FACS analyzer (Becton–Dickinson). For each sample, data from 10,000 volume–gated viable cells were collected (Chapter II–IV).

In the case of Chapter V, 1 x 10^6 cells were incubated with 0.1 ml of each mAb preparation for 30 min on ice in direct immunofluorescence analysis. The dilutions of the FITC–mAbs are as follows: anti–Thy 1.2, 1:400; anti–Lyt 1, 1:100; anti–Lyt 2, 1:100; anti–mouse IgM, 1:100; anti–Thy 1.1, 1:800; anti–L3T4, 1:400. In indirect immunofluorescence analysis, 0.1 ml of 1st antibodies (anti–Mac–1 diluted 1:50 and anti–asialoGM_1 diluted 1:1,000) were added to the cell pellets which were incubated 30 min on ice. After the cell pellets had been washed three times with HBSS containing 0.1% sodium azide, 0.1 ml of FITC–conjugated 2nd antibodies (anti–rat kappa diluted 1:50 and anti–rabbit IgG diluted 1:1,000) were added to the cell pellets. The sample then was incubated for a further 30 min on ice. After the cells had been washed three times, their staining pattern was analyzed using a Becton–Dickinson FACS 440 system and a Consort 30 system. The fluorescence scale was logarithmically amplified. For each sample, data were excluded from analysis by additional staining with propidium iodide.

Cell cycle analysis. Simultaneous immunofluorescence and DNA content analysis was performed as described by Krishan (86) with some modifications. Cells were stained with KBA mAb and avidin FITC, as indicated above. The cells were then fixed on ice in 70% ethanol (stocked at –20°C) for 30 min and subsequently treated with 1 mg/ml of RNAase (Sigma) for 30 min at 37°C. After incubation, the cells were washed with PBS and stained on ice with propidium iodide (50 µg/ml in PBS) for 15 min and analyzed by FACS analyzer as described above.

Depletion of cells by treatment with antibody and complement. Monoclonal antibodies against Thy 1.2 (Serotec clone F7D5), Lyt 1.2 (Cedarlane CG16), Lyt 2.2 (Cedarlane AD4) and L3T4 (GK–1.5), and anti–asialoGM_1 antibody (a generous gift from Dr. K. Okumura, Juntendo University, Tokyo) were used. A cell pellet of 50 x 10^6 cells was treated with 40 µl of antibody solution for 45 min under ice–cooling. The cells were washed twice with PBS and then treated for 45 min at 37°C with

1:8 dilution of guinea pig serum which had been preabsorbed with mouse lymphocytes (nontoxic to thymocytes). The cells were washed 3 times with PBS and used for experiments.

Estimation of frequency of LAK cell precursors. The analysis was carried out according to the limiting dilution method of Lee (87). Various numbers of thymocytes from HC-treated or untreated mice were mixed with equal numbers of mitomycin C-treated (50 μg/ml for 30 min at 37°C) spleen cells and 2,000 JU/ml of rh-IL-2 in Costar No. 3799 U-bottomed plastic plates. After incubation for 7 days at 37°C, the cells in the plates were centrifuged, and a portion (0.1 ml) of the culture medium in each well was replaced by the same volume of a cell suspension containing 4 x 10^3 ^{3}H-uridine-labeled FBL-3 cells. After a further incubation for 18 h, the radioactivity retained in FBL-3 cells was measured by the method described previously (85). Those wells that exhibited larger than 10% specific target cell lysis were scored positive. The percentage of nonresponding wells was calculated by using 24 samples per group. When this (in log scale) was plotted against the number of responding cells (in arithmetic scale), a straight line was obtained as predicted by the zero-order term of the Poisson probability distribution, $P_0=e^{-fN}$ where f=frequency of LAK precursors, N=number of responder cells, and P_0=the probability of obtaining a nonresponding well. When $P_0=e^{-1}=0.37$ (i.e., 37% nonresponding wells, f=1/N). Thus, the reciprocal of the responder cell number corresponding to 37% nonresponding wells is the LAK cell precursor frequency.

Fractionation of LAK cells. A stock (100%) solution of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) was made by adding one part of 1.5 *M* NaCl to 9 parts of Percoll , and was diluted as required with RPMI1640 medium containing 10% FCS. A discontinuous gradient was made in a 10 ml glass tube by layering 2 ml each of 80, 60, 55 and 40% Percoll solutions. An aliquot (1 ml) of a suspension containing 5 x 10^6 lymphokine-activated thymocytes was carefully layered on top of the gradient and the tube was centrifuged for 20 min at 600*g* at 4°C. Cells accumulated at the interface of each gradient solution were carefully pipetted off and washed 3 times with medium.

Assay for conjugate-forming cells. The assay was performed as described previously (10). In brief, test lymphocytes (1 x 10^6) were labeled with 10 μg/ml of azido-fluorescein diacetate (azido-

FDA) (Wako Pure Chemical Industries, Osaka) for 10 min at 37°C. After 3 washing with PBS, the fluorescently labeled test lymphocytes (5 x 10^5) were mixed with target cells (1 x 10^6) in 0.2 ml of FCS medium and centrifuged at 100*g*. After incubation for 30 min at 23°C, the cells were gently pipetted 15 times with a Pasteur pipette. The cells were observed under a fluorescence microscope, and the value of percent conjugate-forming cells (CFC) was calculated by means of the following formula: %CFC = 100 x number of target cell-bound fluorescent lymphocytes / number of all fluorescent lymphocytes.

Effect of KBA mAb on immune responses. KBA mAb was added to various immune response assays as described below and their inhibitory effect on immune responses was examined.

Mitogen response of spleen cells. Mouse spleen cells (5 x 10^5/0.2 ml/well) were cultured with concanavalin A (Con A, SIGMA), phytohemagglutinin-P (PHA-P, Seikagaku Kogyo Co., Ltd., Tokyo, Japan) or lipopolysaccharide of *Escherichia coli* 0127:B8 (LPS, Difco) for 48 h in the RPMI1640 medium containing 10% FCS and 5 x10^{-5} *M* 2-mercaptoethanol (2-ME), and then pulsed with 0.5 μCi/well of [^{3}H]-thymidine ([^{3}H]TdR, Amersham Japan, Tokyo) for 4 h. After incubation, the cells were harvested with a cell harvester (Laboscience Co., Ltd., Tokyo) and the radioactivity retained on glass filters was measured by a standard liquid scintillation technique.

Allogenic mixed-lymphocytes reaction (Allo-MLR). Aliquots (0.1 ml) of the responder cell suspensions (5 x 10^6/ml) were mixed with 0.1 ml of stimulator cell suspensions (5 x 10^6/ml) pretreated with 30 μg/ml of mitomycin C (MMC, Kyowa) for 45 min at 37°C and cultured for 4 days in Costar 96-well plastic plates (#3596) in the RPMI1640 medium containing 10% FCS and 5 x10^{-5} *M* 2-ME. Half the culture media was then replaced with fresh media, and the cells were pulsed with [^{3}H]TdR for 4h.

Autologous mixed-lymphocyte reaction (AMLR). Responder cells were prepared from C57BL/6 mouse spleen cells by passage through nylon wool columns. Nylon adherent cells were detached by teasing the nylon wool in chilled saline solution. These cells were used as stimulator cells after treatment with MMC (60 μg/ml) for 30 min at 37°C. Responder cells (5 x 10^5) and stimulator

cells (5 x 10^5) were mixed in 0.2 ml medium containing 1% fresh normal serum and 5 x10^{-5} *M* 2–ME and cultured for 7 days at 37°C. The cells were pulsed with [^{3}H]TdR for 18 h before harvest. In these experiments, responder cells, stimulator cells, and normal serum were obtained from the same mouse to exclude the xenogeneic factors (88).

Generation of cytotoxic T lymphocytes (CTL) *in vitro*. Aliquots (0.1 ml) of responder cell suspensions (5 x 10^6/ml) were mixed with an equal volume of 2.5 x 10^6/ml of MMC–pretreated (30 μg/ml) stimulator cell suspensions and incubated in a 12–well plate (Costar #3512) in the RPMI1640 medium containing 10% FCS and 5 x10^{-5} *M* 2–ME at 37°C. After 4 days, the cultures were assayed for CTL generation by determining their ability to lyse ^{51}Cr–labeled target cells.

IL–2 production. Mouse spleen cells (5 x 10^6/2 ml/well) were cultured with or without Con A (5 μg/ml) for 18 h. The culture supernatants of Con A–stimulated spleen cells (Con A–sup) were harvested by centrifugation at 400 x *g* for 10 min. The IL–2 activity of Con A–sup was measured using IL–2–dependent T–cell clones, T572 (29, 89) (Chapter IV) or CTLL–2 (Chapter V) cells. These cells did not proliferate with Con A–stimulation alone. In brief, 0.1 ml of CTLL–2 cell suspension was cultured with 0.1 ml of serially diluted Con A–sup for 48 h and then pulsed with [^{3}H]TdR for 4h. Unit of IL 2 was defined as the reciprocal of the dilution that induced 50% of the maximum [^{3}H]TdR uptake by CTLL–2 cells and converted to Jurkat Units (JU) using the reference mouse recombinant IL–2 (r–IL–2, specific activity 3.68 x 10^6 JU/mg protein) produced at Shionogi Research Laboratories.

Acquisition of IL–2 receptors. The acquisition of IL–2 receptors was determined by the development of T–cell responsiveness to IL–2 after short term pulsing with mitogens (90). Mouse spleen cells were cultured with or without mitogens for 8 h, and then washed with either 0.1 *M* α–methyl–D–mannoside or *N*–acetyl–D–galactosamine to remove cell–bound Con A or PHA, respectively. After passing through nylon wool column, the T cells (5 x 10^5/well) were cultured with r–IL–2 (2000 JU/ml) for 36 h in 96–well culture plates. The cells were pulsed with [^{3}H]TdR for 4h, then harvested.

The effect of KBA mAb on IL–2 action. T572 cells (10^4) were cultured with various

concentrations of r-IL-2 in the presence or absence of KBA mAb for 36 h at 37°C. The [^{3}H]TdR incorporation of the cells was measured by pulsing them with [^{3}H]TdR (0.5 μCi/well) for 4 h before the harvest.

Determination of antibody responses.

1) Anti-SRBC antibody responses. Mice were immunized either by a single intravenous injection of a high dose (10^8) or by one or two (at one week interval) intravenous injections of a low dose (10^5) of SRBC. They were bled by heart puncture one week later, and serum total (IgM) and 2-mercaptoethanol (2-ME, 0.1 *M*)-resistant (IgG) hemagglutinin titers were determined.

2) Anti-BSA antibody response. Mice were injected intraperitoneally with a mixture of 1 mg of BSA and 2 x 10^9 of killed Bordetella pertussis organisms (Bp, Nakarai Chemicals). One to three weeks later, they were bled and serum IgE and IgG_1 antibodies were titrated by means of 1-day rat PCA and 1-hour DS mouse PCA, respectively using BSA (1 mg) plus Evans blue (1 mg for mouse and 5 mg for rat) as the elicitor. The antibody titer was expressed as the highest dilution of the serum that gave positive PCA, i.e., blue area with a diameter more than 5mm.

3) Anti-LPS antibody response. Mice were immunized by an intravenous injection of 10 μg of LPS, and bled 4 to 14 days later. Total and 2-ME-resistant antibody titers were determined by agglutination of LPS-coated SRBC according to Rudbach (91).

4) Shultz-Dale reaction. Antigen-induced contraction of uterus of CTS females was tested. The uterus was removed 14 days after an intraperitoneal injection of 1 mg of BSA and 2 x 10^9 Bp. To cause estrus, 0.05 ml of estradiol benzoate (1 mg/ml) dissolved in sesame oil was intraperitoneally injected two days before the uterus preparation. Using a Magnus apparatus, the contraction of uterus muscle was assayed after addition of BSA (final concentration 100 μg/ml).

5) Active systemic anaphylaxis (ASA). Mice which had been immunized to BSA as above described were intravenously injected with 1 mg of BSA. Lethality and shock symptoms were recorded.

Delayed type of hypersensitivity to SRBC. Mice were immunized by a single intravenous

injection of 1 x 10^5 of SRBC (0.2 ml). Four days later, 10^8 SRBC (0.025 ml) and equal volume of physiological saline solution were injected intradermally at their right and left footpads, respectively. Differences in thickness between left and right footpads were measured using a dial gauge 28 and 48 hours after the antigen challenge. Nonspecific swelling induced by the footpad injection in the normal mice was subtracted as the background level.

Skin graft rejection. Tests were done using 5 week old mice. Small pieces of tail skin were removed from CTS, NOD, NON, C3H/He and C57BL/6 mice, and sex-matched mutual transplantation was done by substituting the pieces with the direction of hair growth revealed according to the technique by Festing et al. (92). Acceptance and rejection of the grafts were judged by the presence of hair growth and peeling off, respectively.

Statistical analysis. Student's *t*-test was done to examine the statistical significance of the difference in the anti-SRBC, anti-BSA and anti-LPS antibody titers between the antibody-positive sera of CTS and each reference strain. The analysis was not done, when none of the CTS mice or only one CTS mouse displayed the antibody activity.

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Acknowledgements

I am grateful to Professor Yoshiyuki Hashimoto, the Department of Hygienic Chemistry, Pharmaceutical Institute, Tohoku University, for his valuable suggestions and continued interest throughout the preparation of the present thesis.

I would like to acknowledge Dr. Taichiro Komeno, the ex-director of Shionogi Research Laboratories, and Dr. Masayuki Narisada, the managing director, general manager of Shionogi Research Laboratories for giving this opportunity to accomplish the thesis.

I am deeply indebted to Dr. Takashi Nishimura (Chapter II–IV) and Dr. Minoru Harada (Chapter V–VI) for their worthy suggestions, discussions and collaborations, and also thank Miss Naoko Sato, Mr. So Ohta, Mr. Mitsunobu Matsumoto, Dr. Satsuki Suzuki, and Dr. Susumu Makino for their technical assistances and discussions.

I wish to thank all the members of Immunology Section in Shionogi Research Laboratories for their stimulating discussions and encouragements, and also thank Dr. Ko Okumura and Dr. Hideo Yagita for the generous gift of monoclonal antibodies and anti-asialoGM_1 antibody.

I would like to thank Professor Kazuo Ohuchi and Professor Yasushi Ohizumi for their valuable criticisms and suggestions in revision of this thesis.

I am thankful to my wife, Akiko, my son, Takashi, and my daughter, Michie for moral support.